Calpain Proteinase mRNA and Beta-Agonist Induced Muscle Growth.

by Timothy Parr, B.Sc.

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, May, 1991.

VOLUME CONTAINS CLEAR OVERLAYS

OVERLAYS SCANNED SEPERATELY AND OVER THE RELEVANT PAGE.

Contents.

Chapter 3. Materials and Methods.

Chapter 4. Results.

Chapter 5. Discussion.

Appendices.

References.

List of Figures.

List of Tables.

Abstract.

The mechanism by which β-agonists induce skeletal muscle hypertrophy is believed largely to be through a reduction in protein degradation. These growth promoters are also known to effect the activity of the calcium dependent proteinases (calpains) and their specific endogenous inhibitor calpastatin. The changes in activity appear to be toward a decrease in the calpain system's proteolytic potential. In this study attempts were made to determine whether the altered activity of the enzymes and inhibitor were brought about by induced changes in gene expression, as reflected by altered levels of specific mRNAs.

Various strategies were employed to generate oligonucleotide and cDNA probes to calpain I and II and calpastatin which would detect their respective mRNAs in L.dorsi total RNA samples originating from a bovine growth trial using the β-agonist cimaterol.

Semi-quantiative measurements of specific mRNAs using Northern blot analysis were related to enzyme and inhibitor activities. In addition β-agonist-mediated effects on muscle RNA and expression of actin and myosin light chain 2 mRNAs were determined.

Using a human calpain cDNA specific hybridization was detected for bovine calpain II mRNA, which increased by 34% in the L.dorsi of cimaterol treated animals, similar to the increase in the enzyme activity, 28%.

A novel bovine-specific calpastatin cDNA was generated by the polymerase chain reaction and sequence analysis allowed comparison to those already published for other species. Using this PCR cDNA as a probe multiple calpastatin mRNAs were detected in cattle L.dorsi, as had been observed in rabbit. The predominant mRNA increased by 160% in cimaterol treated steers compared to a 76% change in inhibitor activity.

These changes were in contrast to the essentially unchanged response of muscle total RNA and actin and myosin light chain 2 specific mRNAs in treated animals.

The implications for the calpain system in β-agonist-induced hypertrophy are discussed.

Acknowledgements.

I am indebted to many people for their invaluable assistance and support in the preparation of this thesis. I acknowledge this debt of gratitude to the following people:

To Dr. R.G.Bardsley for his stimulating influence and helpful discussion and suggestions whilst supervising my progress, and his continual help and direction throughout the difficult times when things did not want to work. To my other joint supervisor Professor P.J.Buttery for allowing me to use the resources of the department and his continued support as well as confidence in my ability even when I doubted it.

I would also like to express my gratitude to the following:

Dr R.S.Gilmour of the AFRC Institute of Animal Physiology and Genetics Research, Cambridge, whose advice and practical assistance was invaluable in successfully using the technique of the Polymerase Chain Reaction during course of this project.

Mrs Y.Lasslett for all her help and support, both moral and practical, during the course of the project along with all my colleagues within the Department's laboratories.

All the 'molecular biologists', particularly Mr.A.Hamilton, within the Faculty for their constant helpful advice on practical matters and 'donation' of reagents.

The Ministry of Agriculture Fisheries and Food for funding my studentship.

Members of my family who have given me endless support and encouragement throughout the course of my education especially my parents without whom this work would have been impossible.

Finally the greatest thanks and love go to my wife Teresa who had to put up with all that producing this thesis entailed and Hannah who was the incentive in getting it finished.

Abbreviations

A	ampere.
AMV	avian myeloblastosis virus.
bp	base pairs.
Ci	Curie.
cpm	counts per minute.
cDNA	complementary DNA.
BSA	bovine serum albumin.
DMSO	dimethyl sulphoxide.
ddNTP	dideoxynucleotide triphosphate.
dNTP	deoxynucleotide triphosphate.
dpm	distintegrations per minute.
DTT	DL-dithiothreitol.
EDTA	ethelenediamine tetra-acetic acid (disodium salt).
ε	extinction coefficent.
kb	kilobases.
kDa	kilodaltons.
MOPS	morpholinopropanesulphonic acid.
MMLV	murine Moloney leukaemia virus.
mRNA	messenger RNA.
MW	molecular weight.

Nucleotide bases;

A	adenine.
C	cytosine.
G	guanine.
T	thymine.

rRNA	ribosomal RNA.
SDS	sodium dodecylsulphate.

SDS-PAGE	SDS polyacrylamide gel electrophoresis.
TCA	trichloroacetic acid.
TEMED	N,N,N',N',-tetramethylethylenediamine.
tRNA	transfer RNA.
Tris	tris (hydroxylmethyl)methylamine: 2 amino-2-(hydroxylmethyl) propane-1,3-diol.
UV	ultraviolet radiation.
V	volts
W	watts

Chapter 1. Introduction.

This thesis examines the calcium activated neutral proteinase, calpain, of skeletal muscle and its possible role in muscle growth. Calpain is associated with an endogenous specific inhibitor calpastatin and their activities are ubiquitously distributed in the tissues of mammals where there is differential expression of the calpain isoforms, calpain I and II.

Calpains are involved in particular proteolytic events in cells cleaving membrane, cytoskeletal and contractile proteins along with various enzymes. Calpain does not appear to be a general catabolic enzyme; its role, if any, in muscle protein turnover may be to initiate protein degradation by cleaving specific proteins. As calpain is nonlysosomal, active at neutral pH, and regulated by its own calcium sensitivity it is a good candidate for a trigger thereby beginning a chain of proteolytic degradation events or irreversible 'activation' of proteins. Although there are other proteinases involved in nonlysosomal proteolysis, including pathways that are ATP-dependent and the ubiquitin targeting system which have been described in recent reviews (1,2), this thesis is primarily concerned with the calpains. Subsequent proteolysis, initiated by calpain, could then be taken over by other nonlysosomal or lysosomal systems, the latter being a concentrated environment of several proteolytic enzymes which carry out degradation to a conclusion.

β-Adrenergic agonists act as repartitioning agents by producing an increase in skeletal muscle mass and a decrease in fat deposition in livestock. The means by which they induce muscle hypertrophy is believed to be achieved by suppressing protein degradation resulting in augmented protein accretion. The rate of protein breakdown must be dependent on the activities of the proteolytic enzymes. From observations in our laboratories calpain and calpastatin activity has been associated with this β-agonist induced skeletal muscle growth. Other proteolytic enzymes may be involved but the decrease in protein degradation is concurrent with inhibition of the 'calpain system' by changes in the activity status of the calpain isoforms and calpastatin. The effects of the

inhibition may be to achieve a decline in the initiating events of proteolysis as described above.

Experiments carried out in this thesis were specifically concerned with an examination of this effect, particularly whether β-agonists induce an alteration in gene expression of the calpain system's components which could be responsible for the changes seen in their activities.

Chapter 2. Literature Review.

2.0. Introduction.

In order to comprehend the possible implications of changes in calpain and calpastatin activity in β-agonist induced skeletal muscle hypertrophy an understanding of the calpain system is required. The first sections of this review deal with the structure and the possible activation mechanisms of the calpains along with an examination of calpastatin and its interaction with and inhibition of the enzyme. The identified substrates of calpain mediated proteolysis are then discussed with particular reference to those of skeletal muscle and states of altered protein turnover where the calpain system is thought to be involved.

A brief review of β-agonist induced skeletal muscle hypertrophy and an appraisal of studies carried out so far on the relationship of the calpain system to this type of muscle growth is discussed. Finally there is an examination of the possible means by which β-adrenergic agonist-mediated induction of cAMP could bring about increases in the activities of enzymes and/or protein expression with particular reference to the calpain system.

2.1.0. The Calpains.

Calcium activated neutral proteinases (CANP), also known as calpains, are intracellular nonlysosomal thiol (cysteine) proteinases. They have a neutral pH optimum and are absolutely dependent on calcium ions for proteolytic activity. There are at least two isoforms of the enzymes with different calcium sensitivities:

Calpain I (μCANP) activated at μM concentrations of Ca^{2+}.

Calpain II (mCANP) activated at mM concentrations of Ca^{2+}.

Early purification procedures identified an isoform of calpain from pig skeletal muscle (3) which required 0.57mM Ca^{2+} for half maximal activity. This is now called calpain II and has been isolated from many tissues across mammalian species and been identified in fish (4) and crab muscle (5). The second isoform of calpain, calpain I, was

first isolated in canine cardiac muscle and has subsequently been purified in other species (6,7,8). The distribution of calpain is probably ubiquitous in mammalian tissues and cells but the activity of the two isoforms varies (9,10). In human and rat erythrocytes only a calpain I isoform was identified by Murachi et al (9), although it was called calpain II by Pontremoli et al (11). With a half maximal activity at a Ca^{2+} concentration of 40-50 μM the erythrocyte calpain would be calpain I by current definition as native calpain II is not active at these calcium concentrations.

In some tissues, like chicken skeletal muscle, where only a calpain II type isoform was reported to be present (7,12), it has subsequently been found that there are probably three isoforms. They were identified by their calcium requirements for activity as a calpain I, calpain II and a 'high' calcium requiring isoform (13). Such variation in the distribution and activity of the calpain isoforms may be, in part, due to the presence of the specific protein inhibitor to calpain, calpastatin, and the autolytic change in the isoforms, which alter their calcium sensitivity, causing stimulation but ultimately lead to a loss of activity.

Full understanding of the calpain isoforms will emerge as the genes are sequenced and the activation characteristics along with the physiological roles of the isoforms are described.

2.1.1. The Subunit Structure of Calpain.

Both calpain I and II are composed of 2 subunits to give a heterodimer consisting of approximately 80 kDa and 30 kDa proteins in each isoform. The 80 kDa large subunit contains the catalytic capability. The primary sequence predicted from cDNA analysis was first determined for the chicken skeletal muscle large subunit (14). Subsequently cDNAs for the calpain I and II large subunits from rabbit and human have been isolated (15-17). The cDNA sequence of 30 kDa small subunit has also been determined for pig, rabbit, human and cattle (18-21). The small subunit is common to both isoforms of the enzyme (22). It follows that the calcium sensitivity of calpain isoforms is attributable to their large subunits.

The 80kDa large subunit.

Both calpain I and II contain the same domain structure in their large subunits, as shown in Figure 1 (23). The large subunit consists of four domains. Domain II contains the proteinase activity, identified as the cysteine proteinase type in nature from its homology[1] to the similar enzymes cathepsin B, H and papain (14). The amino acid residues responsible for the catalytic activity are cysteine and histidine at positions 108 and 265 in the primary sequence. Domain IV has been identified as the 'calmodulin-like' Ca^{2+} binding domain, as it contains E-F hand motifs which bind Ca^{2+} (24), similar to those in sequences like calmodulin, troponin C and myosin light chain (14). Four E-F hands, each a helix-loop-helix structure, were identified in the chicken skeletal muscle calpain and the other isoforms which have been isolated. Domains I and III have no homology with any other known protein sequence (23).

Comparison of primary sequences of chicken calpain with both isoforms of human have shown a high homology across isoforms and species in domain II (Table 1). Domain IV has variation in sequence homology, which is expected as the domain is responsible for the calcium sensitivity. Domain III is homologous across the isoforms. It is suggested that domain III links the calcium binding characteristics of domain IV to the catalytic properties in domain II (17). Domain I, the amino terminal, differs in size and homology between calpain isoforms (Table 1). This domain is involved in the autolytic activation of calpain so homologies may indicate similar activation mechanisms.

The 30KDa small subunit.

The small subunit is also divided into distinct domains (Figure 1). The N-terminal domain V is rich in glycine residues and is believed to be involved in membrane interaction as it is hydrophobic in nature (25) (Figure 1). The C-terminal domain IV' is similar to domain IV in the large subunit (19,23) in that it contains four proposed E-F hand structural motifs. From site directed mutagenesis studies on the small subunit domain IV' the capacity and position of the active calcium binding E-F hands of both domain IV' and IV have been identified (26). Of the eight potential sites five are thought to be capable of binding calcium ions. This had been confirmed in earlier studies where

[1] The word 'homology' is used to indicate that the similarity of the nucleotide and deduced amino acid sequences of a group of proteins is due to them evolving from a common ancestral gene.

Figure 1: The domain structure of calpain (23).

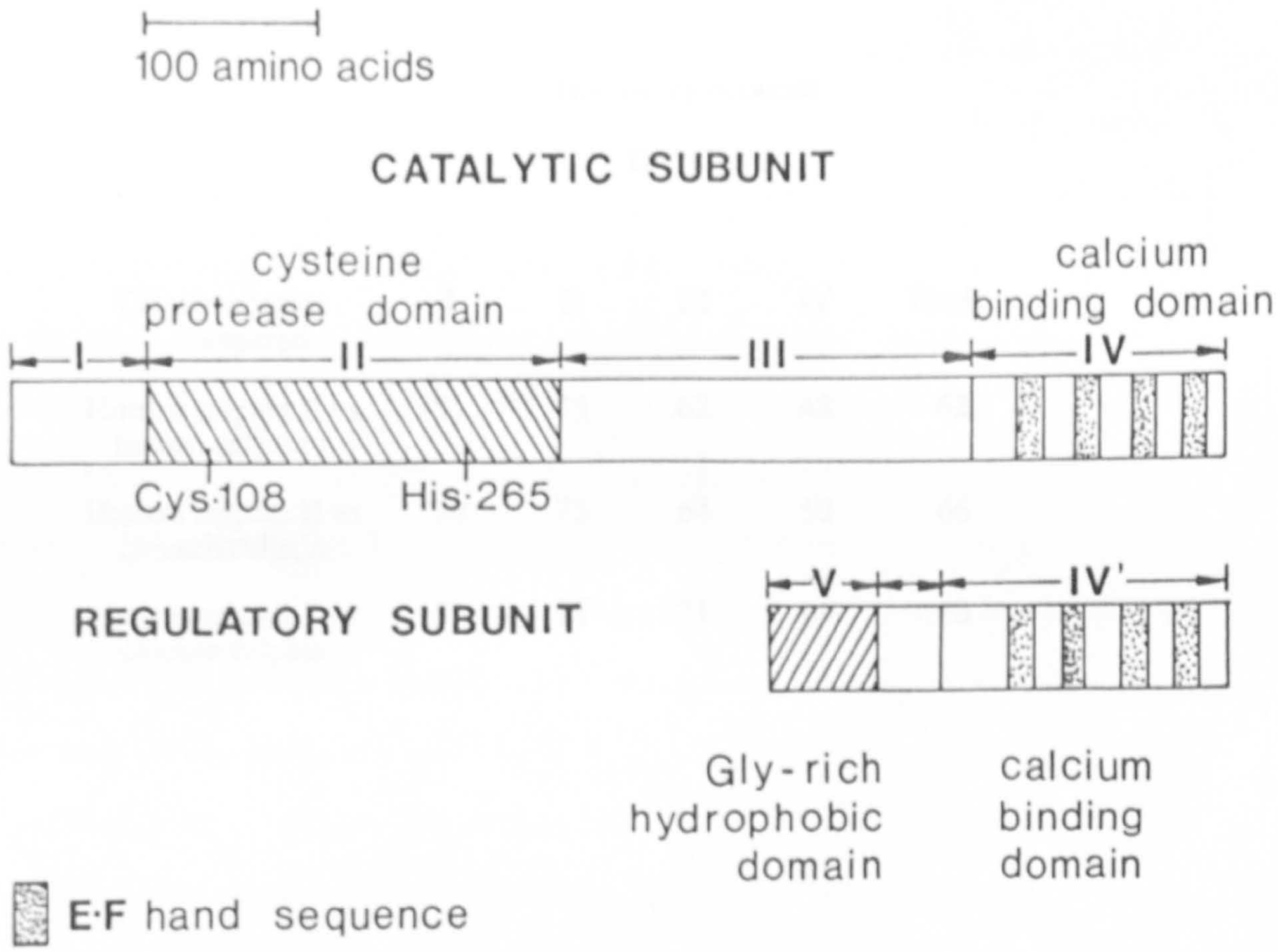


Table 1: Amino acid homology among the calpain large subunits of chicken (14) and the two isoforms of human (16,17). Taken from Imajoh et al (17).

Calpain species compared	Homology between Domains I	II	III	IV	Total
Human calpain II vs human calpain I	51	73	62	48	62
Human calpain II vs chicken calpain	70	75	64	53	66
Human calpain I vs chicken calpain	51	78	71	65	70

in chicken calpain 5-6 moles calcium ions bound per mole of calpain (27). Three of the sites are occupied in the large subunit and two in the small. Domain IV' has also been identified as the point of association between the large and small subunit.

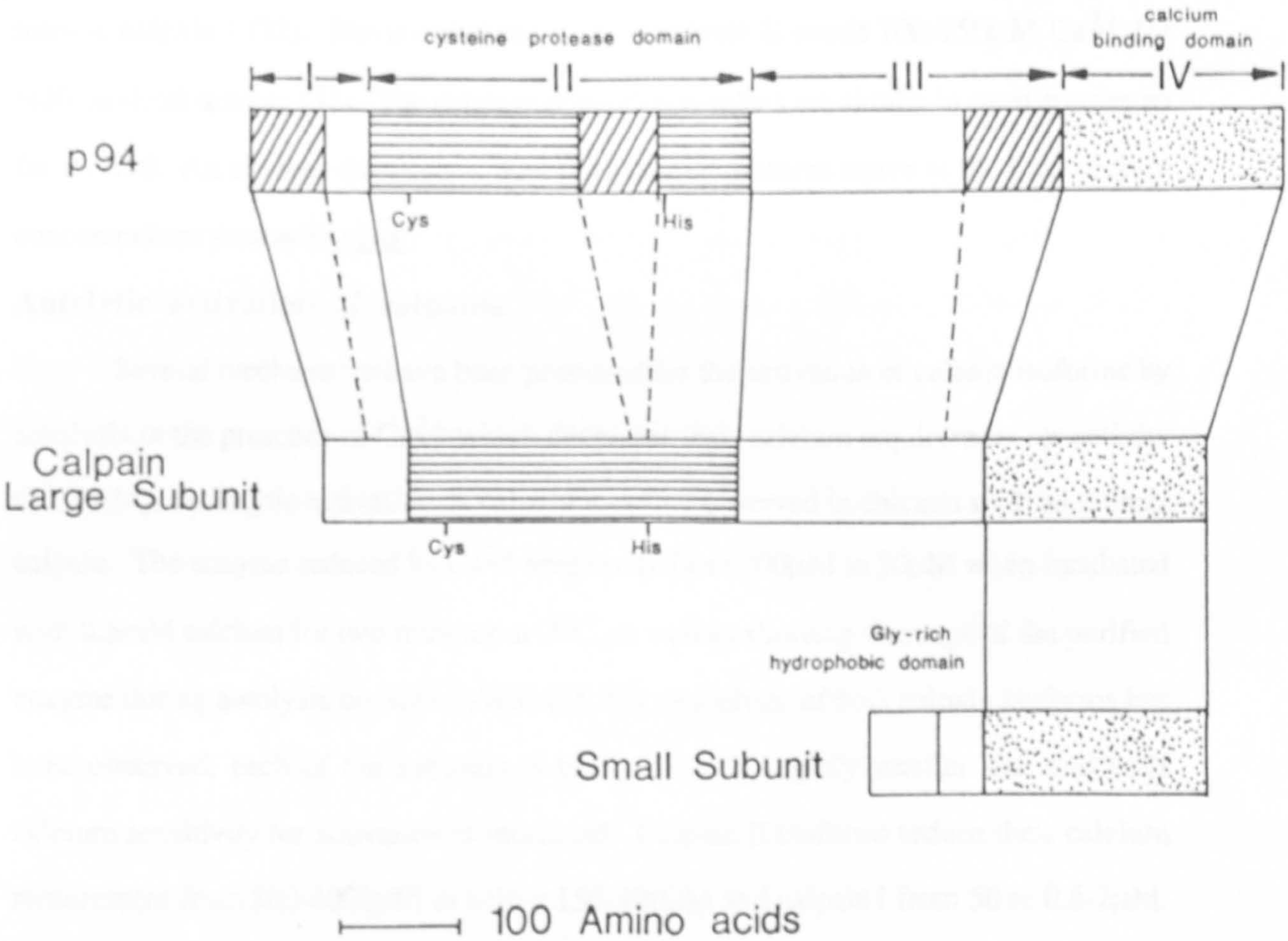
The small subunit is clearly involved in the calcium binding of the enzyme, but is identical in both isoforms (22). Association of calcium ions to the small subunit may trigger some conformational change in the large subunit leading to its conversion to an active form as the calcium sensitivity of the enzyme appears to be attributable to the large subunit.

p94

Calpains appear to be ubiquitous in their tissue distribution although the isoforms do vary in their expression. In addition a calpain-like protein p94 encoded by a recently isolated cDNA from rat and human has been identified as being specific to skeletal muscle (28). The sequenced cDNA encoded a protein of 821 amino acids of calculated molecular weight 94kDa, which was homologous to calpain I and II large subunits, 54% and 51% respectively. Sorimachi et al (28,29) suggested that the protein was calpain large subunit-like and that it could be divided into four domains with cysteine proteinase and calmodulin-like binding regions, domain II and IV. It is not known whether p94 is associated with the small subunit like the other isoforms. The predictions of the 'calpain-like' nature of the protein was based on its cDNA sequence but it has yet to be isolated as a functional calcium sensitive proteinase.

The predicted primary sequence of p94 does contain novel stretches which are inserted into the calpain sequence in domains I, II and III (Figure 2). Those into domain II and III may affect the possible proteolytic function of the putative enzyme (29). The domain II insert between the cysteine and histidine residues involved in enzyme activity may prevent proteinase action. Interestingly the insertion into domain III has some primary sequence homology to α-actinin and dystrophin (29), so that it may have binding capability to other proteins . There is also a sequence which is homologous to one in domain V of the small subunit and thought to be involved in the autolytic cleavage of this domain. Cleavage in this region of p94 could mean that the enzyme could become

Figure 2: The proposed domain structure of p94 (29).



dissociated from its calcium binding domain, thereby becoming constitutively active.

The skeletal muscle-specific expression of p94 is of particular interest, if it is a calpain-like proteinase, as protein turnover is an important metabolic function in the tissue and a regulating proteinase may have critical role in muscle growth and metabolism.

2.1.2. Activation of Calpain

Physiological free calcium concentrations within cells are thought to be in the micromolar range or less (300-1200nM). For calpain I the calcium concentration required for half-maximal activation is in the range 45-50μM Ca^{2+} for bovine skeletal muscle calpain I (32). Bovine skeletal muscle calpain II needs 700-750μM Ca^{2+} for half-maximal activity (32). The calcium dependence values are similar in most species so far studied. An obvious question is how does calpain become active at the low calcium concentrations present <u>in vivo</u>?

Autolytic activation of calpains.

Several mechanisms have been proposed for the activation of calpain isoforms by autolysis in the presence of Ca^{2+} which decreases their calcium requirement for activity (23,30-34). Autolytic activation of calpain was first observed in chicken skeletal muscle calpain. The enzyme reduced its Ca^{2+} sensitivity from 400μM to 30μM when incubated with 0.5mM calcium for two minutes at 0°C, as well as showing cleavage of the purified enzyme during autolysis on SDS-PAGE (35,36). Autolysis of both calpain isoforms has been observed; each of the subunits is truncated to a slightly smaller size and their calcium sensitivity for activation is increased. Calpain II isoforms reduce their calcium requirement from 500-1000μM to below 150-100μM and calpain I from 50 to 0.6-2μM. During autolysis the small subunit appears to undergo the same pattern of degradation in both isoforms as determined by SDS-PAGE. Approximately 90 amino acids are removed from the N-terminal domain V, the molecular weight is reduced by 30% to 18-20kDa. The autolysis of the large subunits does not appear to be the same in both isoforms. Calpain II is not as noticeably reduced in size as calpain I which is cleaved to approximately 76kDa as determined from SDS-PAGE (30,31). In the rabbit skeletal

muscle isoforms 27 amino acids are removed from the N-terminus of calpain I, whilst 19 are cleaved from calpain II (30). It is the removal of the N-terminal fragment from the 80kDa subunit which gives the change in calcium sensitivity (37).

Autolysis leads to decreased stability of the subunits. Prolonged incubation of calpain II with high levels of calcium (mM) leads to its eventual degradation with loss of activity (38). Autolytic activation could alternatively be viewed as the first step in the removal of calpain activity and a means by which prolonged calpain-mediated proteolysis is avoided.

The effects of phospholipids on autolysis.

Although the modification of the N-terminus of the small subunit appears not to affect the changes in calcium sensitivity of the isoforms (37) it does have a function in the autolytic activation. Autolysis of calpain II has been shown to take place at lower calcium concentrations than normally required in the presence of a specific phospholipid. A reduction from 680μM to 87μM calcium for half maximal rate of autolysis of bovine smooth muscle calpain II is seen in the presence of 50μM phosphatidylinositol (39). This effect is negated when the N-terminus of the small subunit is removed, so that it must be essential for the association of calpain II to the phosphatidylinositol (40). In liposomes of the phospholipid used for these experiments the binding of calpain II does not require calcium (30,41). However calpain association to biological membranes is reported to be induced in the presence of calcium (30,42,43). Most of these observations are on calpain I (42,43). Increases in calcium concentration (1-2μM) lead to membrane association of the enzyme where it is autolysed to a low calcium requiring form in the lipid environment (42,43). The association of the erythrocyte calpain I to the membrane led to an increased degradation of the membrane proteins such as spectrin (43). The effect of calcium on the calpain II association to biological membranes has not been studied to the same extent. One of the reasons is that the cells used for the observations are relatively easily isolated single cells, such as erythrocytes, which only contain the calpain I isoform.

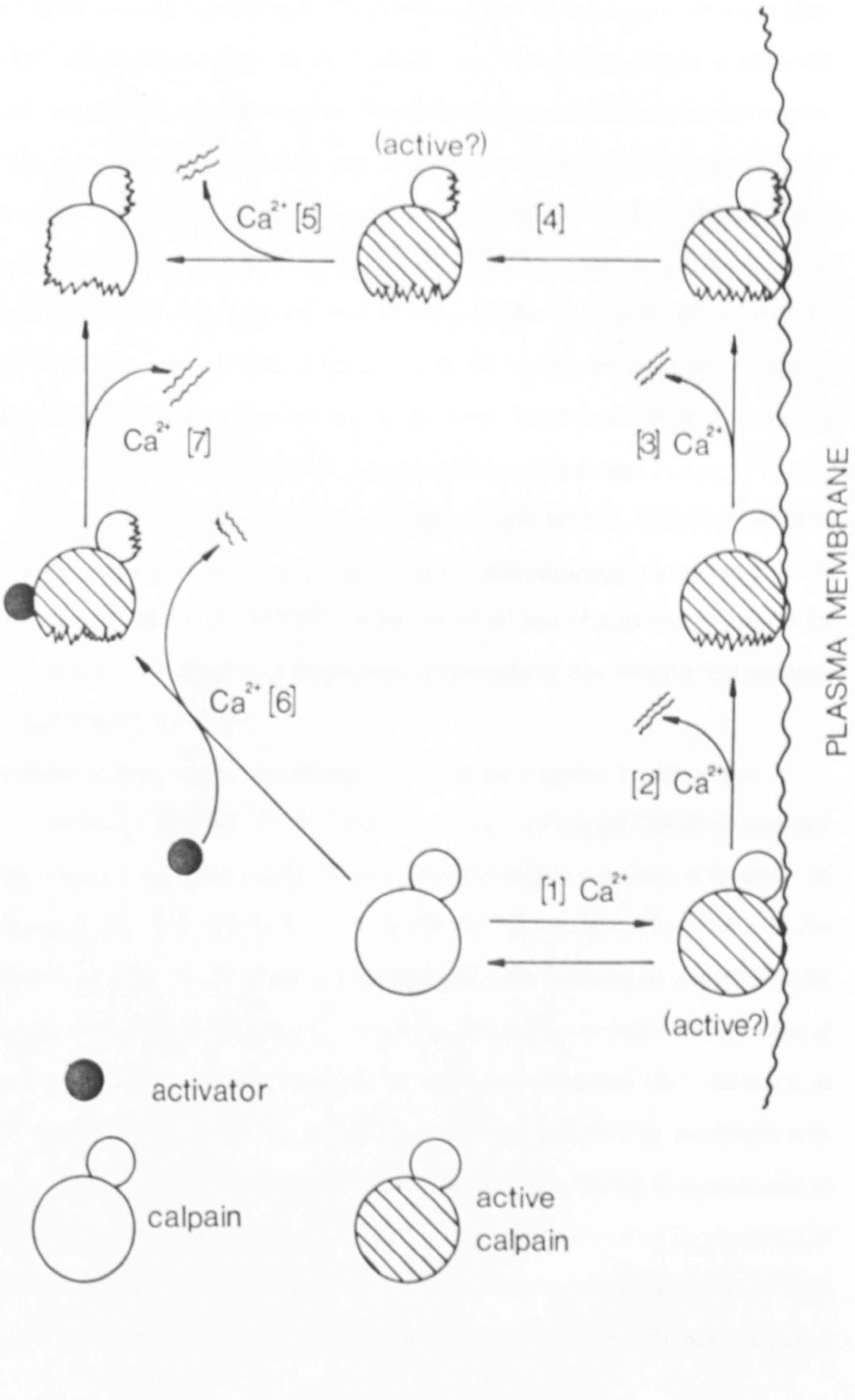
A proposed mechanism for the activation of calpains.

This was based on the above observations and a proposed mechanism suggested by Mellgren (49) is described in Figure 3. From in vitro measurements cytosolic calpain would not be active at the intracellular free Ca^{2+} concentration, especially calpain II. The enzyme may associate with the phospholipids in the membranes via the N-terminus of the small subunit, in a calcium dependent step [1]. Autolysis of the large subunit then takes place in the lipid environment at lower calcium concentration than required in the cytosol, leading to activation of the enzyme at the membrane [2]. Dissociation from the membrane is achieved by autolytic cleavage of the N-terminal glycine-rich hydrophobic domain in the small subunit [3] and [4]. This autolysed cytosolic calpain may be active. However the intracellular free Ca^{2+} concentration is possibly not high enough for activation of autolysed calpain. Inactivation of the enzyme is caused by further autolytic fragmentation [5].

Cytosolic autolysis of calpain II is unlikely to occur as the Ca^{2+} concentrations required are believed to be well above those which are physiological - the mechanism for the activation of cytosolic calpain I is different and is dealt with in a subsequent section. In the presence of cytosolic 'activators', which lower the calcium requirements for activation, calpain may autolyse and become active in this environment [6]. The activators appear to be specific to calpain II (45-48) and have been variously identified as proteins (45,46), small peptides (47) and a catabolic product of L-leucine, isovalerylcarnitine (48). The ability of the activators to reduce the calcium sensitivity of calpain II varies. Some are unable to achieve a high reduction in calcium dependence (47) so either role in the activation scheme of calpain is questionable.

Further autolysis of the enzyme probably results in inactivation [7].

Figure 3: A model for the activation of calpain.



The proenzyme theory of calpain activation.

Based on the model above it has been suggested that isolated calpains are essentially proenzymes which require autolysis for activity, especially calpain II (30). Incubation of calpain II in the presence of millimolar calcium and substrate results in the autolytic degradation and a decrease in the calcium concentration required for activity (32). The calcium concentrations required for the assay of calpain II activity and also for autolysis, are equivalent. Acccording to the proenzyme theory the calpain is being converted to its 'active' form at the calcium concentration present, and then is in an environment where it can be active as a proteinase (30). It is difficult to prove the proenzyme theory. This might be done by careful kinetic analysis of the rate of production of proteolytic fragments from the substrate. There would be an expected lag phase of cleavage whilst the proenzyme is converted to an active state.

For calpain I the proenzyme theory is less clearly defined. Calpain I has been shown to be active at calcium concentrations below that required for autolysis (31,32). Bovine calpain I requires 50μM Ca^{2+} for half maximal rate of activity and 210μM for half maximal rate of autolysis. In the presence of phosphatidylinositol the half maximal rate of autolysis was at 150μM.

The effects of membrane associated proteins on calpain I activation.

A different mechanism for the autolytic activation of calpain I has been proposed with the enzyme using membrane proteins as specific binding substrates (33,34,44). In erythrocytes calpain I association to 'inside-out' membranes does result in the degradation of proteins identified with membranes. The binding of calpain I to the erythrocyte membranes is thought to be via membrane proteins, as proteinase digestion of inside-out membranes reduces the degree of calpain I association (44). Removal of phospholipid by digestion with phospholipases C and A_2, as well as incubation with Triton X-100 failed to affect the calpain I binding (34,44). The lack of a large decrease in Ca^{2+} required for autolysis in the presence of phosphatidylinositol in vitro (32) of calpain I (unlike calpain II) may be because the autolytic activation of calpain I is achieved through a different mechanism. The binding of calpain I to membranes is calcium

sensitive (33,43), whereas this effect on calpain II is not known. Autolysis of calpain I does take place on phospholipid depleted membranes at micromolar calcium concentrations (1.4μM), although changes in enzyme activity were not assessed (34).

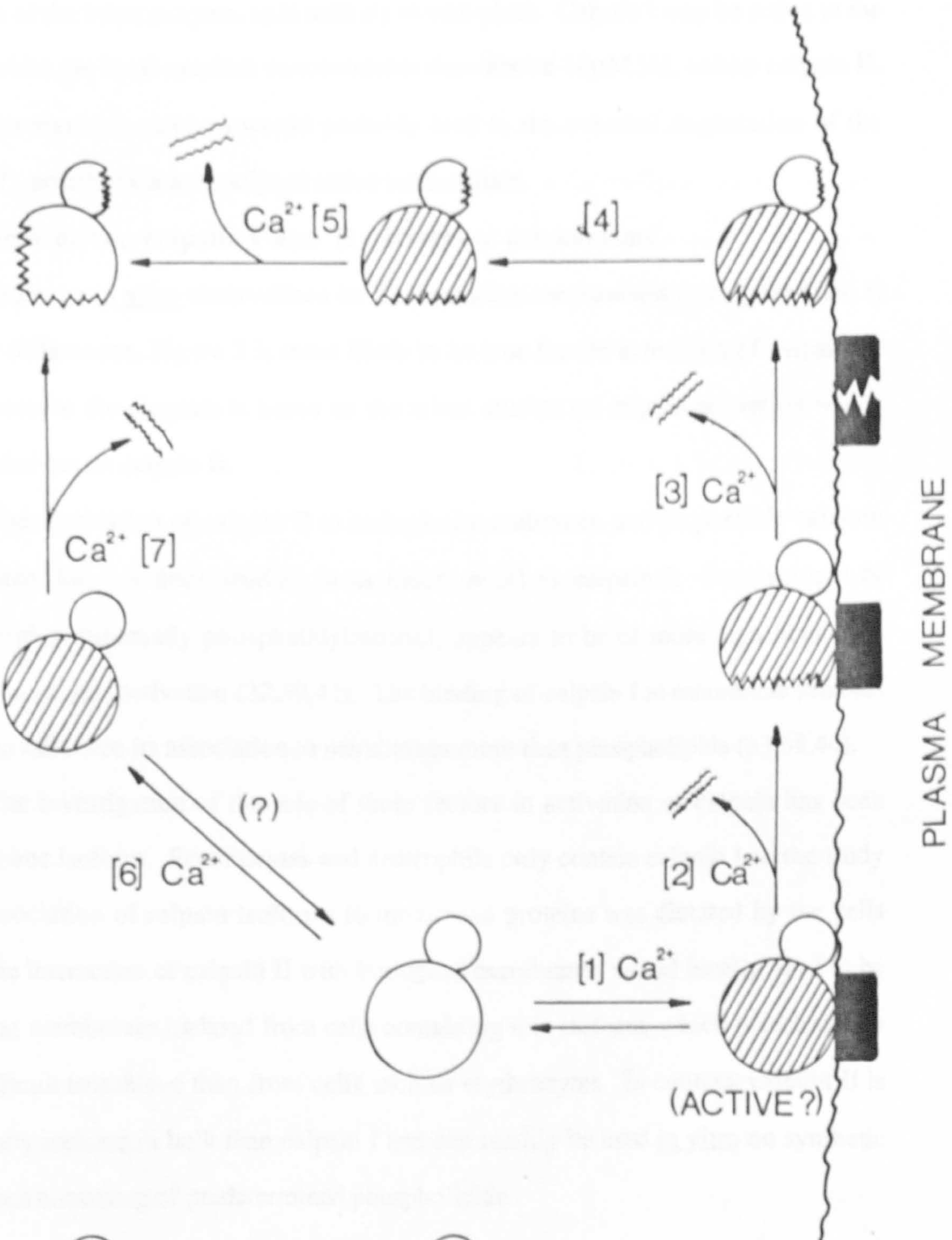
It can be concluded that calpain I binds via membrane proteins to 'inside-out' vesicles (33,34,44). Proteins which are cytosolic substrates for calpain I and also the inhibitor calpastatin might be expected to inhibit the binding of calpain I to the membrane. Whether a truncated calpain I trimmed small subunit with no N-terminus will still bind to biological membranes through this membrane protein/substrate association has yet to be reported. Cytosolic protein 'activators' have been identified for calpain II (45,46), as yet none of these 'activators' have been isolated for calpain I. Membrane associated calpain I 'binding proteins' maybe similar to these 'activators'.

Since calpain I binding to membranes has been linked with subsequent degradation of membrane proteins (33,43), autolytic activation by this mechanism would almost inevitably result in membrane damage. However the requirement for a rise in calcium concentration for the binding may be a critical factor in regulating this mechanism. A technical consideration may be that in the system studied the membranes have been damaged, thereby exposing the membrane associated proteins to unphysiological proteolytic cleavage in vitro.

A proposed mechanism for calpain I activation.

From the in vitro evidence discussed above calpain I activation in vivo may be different to the mechanism proposed in Figure 3. Figure 4 illustrates a possible activation scheme for calpain I. It associates with membranes and this is dependent on increased Ca^{2+} levels. The association and subsequent activation may be through phospholipid interaction with the small subunit, but membrane proteins which act as binding/substrate sites may also be involved [1]. Activation of the membrane associated calpain I in the presence of calcium involves autolysis of the large subunit [2]. This active form then may degrade its associated binding protein. Binding could be maintained by the N-terminus of the small subunit, cleavage of this region [3] leading to the dissociation of the membrane autolysed form [4] which would be active. Further autolysis leads to

Figure 4: A model for the activation of calpain I.



inactivation [5].

Autolysis of calpain I in the cytosol requires higher calcium concentrations than activation of the intact enzyme, so is unlikely to take place. Calpain I may be active in the cytosol when the local calcium concentration rises above 10μM [6], unlike calpain II. Further increases in calcium would probably lead to the eventual degradation of the calpain [7], possibly via an autolysed active intermediate.

Differences in the calpain I and II activation mechanisms.

From the in vitro observations on the activation mechanisms of calpain I and II and their differences, Figure 3 is more likely to be true for the activation of calpain II. This is because the diagram is based on the initial studies on calpain activation which were carried out on calpain II.

The association of calpain II to biological membranes, and its possible calcium dependence, has not been studied in as much detail as calpain I. Involvement of phospholipids, especially phosphatidylinositol, appears to be of more significance in calpain II autolytic activation (32,39,41). The binding of calpain I to membrane proteins appears to influence its association to membranes more than phospholipids (33,34,44).

The investigation of the role of these factors in activation of calpain has been biased to one isoform. Erythrocytes and neutrophils only contain calpain I so the study of the association of calpain isoforms to membrane proteins was dictated by the cells used. The interaction of calpain II with biological membranes would ideally need to be studied on membranes isolated from cells containing this isoform, which is technically more difficult to achieve than from cells such as erythrocytes. In contrast calpain II is more easily isolated in bulk than calpain I and can readily be used in vitro on synthetic membranes consisting of predetermined phospholipids.

2.1.3. Summary

Although the proposed proenzyme theory of activation for calpain has been questioned by some groups working in the field, cytosolic calpain II would still seem to require a reduction in its calcium requirement for activation to be functional at physiological calcium levels, even if it is active in its unautolysed state. From the work of Cong et al (32) calpain I could be active without the need for autolysis, but an increase in calcium sensitivity would be required for full proteolytic activity. Local fluctuations of calcium concentration may reach the lower range needed for native calpain I activation.

Autolysis is the means by which both calpain I and II can be active at lower calcium concentrations than their unautolysed states. In vitro studies have shown autolysis does require higher than physiological calcium concentrations. By associating to membranes both isoforms can achieve autolysis at reduced calcium concentrations, but the mechanism by which the two enzymes bind to the membranes appears to be different. Activity in the cytosol is also affected by factors which are apparently isoform specific, such as the calpain II-specific activators. These different activation mechanisms for each isoform may indicate a different role for their proteolytic action.

2.2.0. Calpastatin.

Calpastatin is a highly specific endogenous inhibitor to both isoforms of calpain. It was discovered in rat liver and was reported to be associated with and inhibitory to calpain activity (50). Calpastatin has subsequently been identified in various tissues but as with calpain the activity of the inhibitor varies. In skeletal muscle there is usually more calpastatin than the combined activity of calpain (9). This again raises the question of how can calpain ever be active in muscle cells ? This will be considered at a later stage in the review, after first describing the characteristics of the inhibitor.

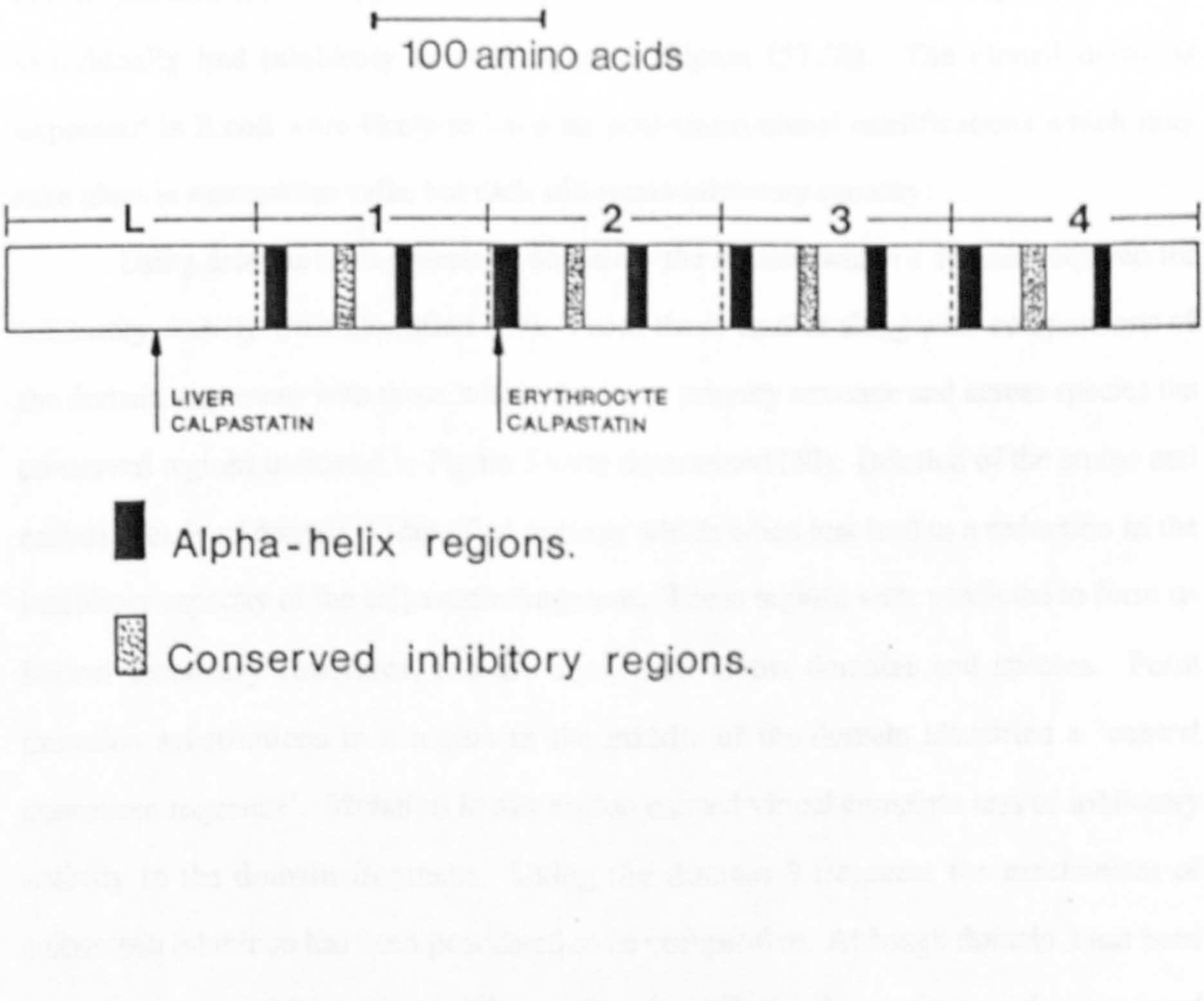
2.2.1. The Structure of Calpastatin.

When isolating calpastatin care has to be taken as the inhibitor is very susceptible to proteolytic cleavage (50,51). This has led to confusion concerning the exact size of the inhibitor as determined by SDS-PAGE and its stoichiometry of inhibition, which ranges from 3-10 mol calpain/mol calpastatin dependent on the tissue from which it was isolated (30). It seems clear however that a mole of inhibitor will inhibit more than one mole of calpain (30). From purification studies calpastatin has been classified into two groups, liver and erythrocyte types. The erythrocyte type is smaller at 68-79kDa (4,30), whilst the liver calpastatin is 107-170kDa (30).

The primary structure of the inhibitor has been determined from the cDNAs isolated from rabbit liver (52), pig cardiac muscle (53) and human liver (54) calpastatin mRNA, the three consisting of 718, 713, 698 amino acids respectively.

The primary sequence of calpastatin can be divided into five domains, domain L and domains 1-4 each being approximately 140 amino acids long. The domains 1-4 are mutually homologous and have been identified as containing the inhibitory capacity (Figure 5). The calculated molecular weight for pig calpastatin is 77kDa, whilst its molecular weight determined by SDS-PAGE gave a value of 107kDa. This discrepancy between the calculated and the observed molecular weight may be due to post-transcriptional modification. Such modification was identified in the erythrocyte inhibitor where N-terminal analysis showed that it was a truncated form of the full length protein.

Figure 5: The domain structure of calpastatin (53). The diagram shows the predicted structure of rabbit calpastatin with the positions of the starting residue of the liver and erythrocyte inhibitors.



The erythrocyte inhibitor starts at residue 290, whilst the liver calpastatin starts at residue 80 of the cDNA encoded primary sequence (30), shown in Figure 5. The erythrocyte inhibitor thus lacks one of the inhibitory domains, whilst the liver calpastatin contains all four domains, the L domain being truncated. It is proposed that calpastatin is further post-transcriptionally modified to cause the anomalous behaviour on SDS-PAGE which gives a larger estimation of calpastatin size (30). Uemori et al have suggested that because calpastatin is rich in proline residues it is this which affects its mobility on SDS-PAGE (56).

Identification of the peptide sequence in calpastatin responsible for inhibition of calpain.

The two groups, led by Suzuki and Murachi, involved in producing the cDNAs for calpastatin have tried to identify whether each of the homologous domains individually had inhibitory activity against calpain (57,58). The cloned domains expressed in E.coli were likely to have no post-translational modifications which may take place in mammalian cells, but each still retain inhibitory capacity.

Using deletion mutagenesis of domain 3 the regions within a domain required for inhibitory activity were identified (59). From these studies along with comparisons of the domain sequences with those within the same primary structure and across species the conserved regions indicated in Figure 5 were determined (60). Deletion of the amino and carboxyl ends of domain 3 identified regions which when lost lead to a reduction in the inhibitory capacity of the calpastatin fragment. These regions were predicted to form α-helical secondary structures, and are conserved across domains and species. Point mutation substitutions in a region in the middle of the domain identified a 'central consensus sequence'. Mutation in this region caused virtual complete loss of inhibitory activity in the domain fragment. Using the domain 3 fragment the mechanism of calpastatin inhibition has been postulated to be competitive. Although domain 3 has been identified as one of the weaker inhibitory domains (60) the observations on the conserved inhibitory sequence have been confirmed in domain 1 of the calpastatin sequence (56,61). The assertion that calpastatin is a competitive inhibitor has also been confirmed using a

synthetic peptide from domain 3 (60).

Subsequent work on the central consensus region has tried to identify the minimal amino acid sequence required for inhibitory activity against calpain. Such a peptide could potentially be used as a specific inhibitor against calpain proteolysis. Both Suzuki's and Murachi's groups have used synthetic peptides and deletion mutations to determine the minimal consensus sequence with inhibitory capability (56,59-61). When a short peptide sequence of up to 34 residues was used the inhibition tended to be greater against calpain II than calpain I (60), whilst when the complete domain was used the inhibition of the isoforms was reversed (59). This suggested that calpain I requires the α-helical regions at the amino and carboxyl termini. The peptide fragments do not contain these sequences.

Both Suzuki's and Murachi's groups agree that the inhibitory peptide is based on the sequence;

Thr-Ile-Pro-Pro- X -Tyr-Arg

where X can be variable. However this peptide sequence alone is not sufficient to inhibit calpain activity.

Suzuki's group (60) suggested the basic inhibitory sequence is;

R E
L G X K D X T I P P X Y R X L L

Whilst Murachi's (56) group have suggested a 'M-sequence' of;

R E
L G X K D X T I P P X Y R

which lacks three C-terminal residues of Suzuki's sequence. Murachi et al (56) considered this sequence to be the basic one required in inhibitory peptides. The shortest peptide with the capability of 50% inhibition of calpain was of 18 residues based on the above sequence (56). However all the peptides have lower inhibitory activity than the full length domain fragment (60).

2.2.2. The Interaction of Calpastatin with Calpain.

The interaction of calpastatin with calpain requires calcium in vitro. The calcium required for binding is thought to interact with calpain as calpastatin contains no identifiable calcium binding E-F hand sites (52-54). Kapprell and Goll (62) have shown that both unautolysed and autolysed isoforms of calpain have different calcium requirements for their association to calpastatin, in vitro. Their observations revealed that the free calcium concentration required for half maximal association of calpain to calpastatin is less than that needed for half maximal proteolytic activity of autolysed and unautolysed calpain II and autolysed calpain I. For unautolysed calpain I the half maximal calcium concentration for enzyme activity was 34μM, whilst the half maximal binding of calpain I to calpastatin was at 42μM calcium. The native calpain I could be active in the range of 10-34μM whilst the other forms would be inactive in the presence of calcium and calpastatin. This study suggested that autolysed forms of calpain, although they have a greater calcium sensitivity for activity than the unautolysed isoforms, would not be active unless calpastatin was removed from their environment. However in vivo the interaction of calpain with calpastatin may be affected by factors similar to those affecting calpain and its autolysis. The involvement of biological and artificial membranes was not investigated in the study, but this interaction is known to have a critical effect on calpain activity as described in section 2.1.2. (30,33,39,40). The question might be asked is calpastatin an effective inhibitor in this membrane environment ?

Calpastatin has been isolated associated to the sarcolemma of cardiac muscle (63). The affiliation is thought to be through the L-domain of the inhibitor as proteolytically cleaved calpastatin lacking this domain does not associate with phospholipids (64). The erythrocyte type inhibitor, which lacks domain L (30), does not bind to the erythrocyte membrane nor does it bind to phospholipids (63). The L domain interaction is believed to be through the hydrophobic and basic amino acids which would associate with phospholipids reported to be 'acidic', phosphatidylinositol /serine, but not with the so called 'neutral' ones, phosphatidylcholine/ethanolamine (64). Although calpastatin does

appear to bind to membranes the effect of this on calpain's activity at the membrane has not been investigated,

Autolysis and calpastatin cleavage in the calpain-calpastatin complex.

Calpastatin complexed to calpain I and II was shown to be fragmented even when there was sufficient inhibitor present to cause complete inhibition of the enzyme (65). This suggested that calpastatin was a suicide inhibitor/substrate of calpain This had been observed earlier by Mellgren et al (66), although they suggested that excess calpain was required for the degradation of calpastatin. Nakamura et al (65) also found that calpain underwent autolysis in the calpain-calpastatin complex. However these observations were made using high calcium concentrations above those required for half maximal binding of calpain to calpastatin in vitro, which is below that needed for half maximal activity of the calpain II isoform. The calcium concentrations used for experiments on calpain I and II binding to calpastatin were also above those at which full autolysis of the isoforms had been observed, 250μM and 6mM respectively (see section 2.1.2.). Mellgren et al have suggested that autolysis did not take place in the complex (66), but in their study the observations were from SDS-PAGE which may have not been loaded with sufficient protein to see the autolytic fragments. Nakamura et al (33) have also reported that calpastatin will inhibit the binding of calpain I to membranes. However they reported that calpain I associates to membranes at approximately 1μM (33), well below the half maximal binding value determined for native calpain I association to calpastatin in vitro which was 42μM, in the absence of membranes (62).

How does calpain become active in vivo in the presence of calpastatin ?

Although from Kapprell and Goll's study (62) on calpain-calpastatin binding and its calcium dependence it would appear that most of the calpain activity both native and autolysed is inhibited in vitro, native calpain I was active at a Ca^{2+} range which could be attainable by local fluctuations of calcium i.e. 10-34μM Ca^{2+}. They also acknowledged that in vivo there may be additional factors that reduce the calcium concentration for calpain activity below that required for their binding to calpastatin in vitro. Such factors

could also be responsible for calpain II activation at physiologically attainable levels which otherwise appear to negate its activity in vivo.

Such factors or activators were described earlier having been isolated by Pontremoli et al, and appear to have their effects chiefly on calpain II (45,46,48). Some of the activators are capable of lowering the inhibitory capacity of calpastatin against calpain II in vitro (45,46). Such activators in vivo may combine activation of calpain II and repression of calpastatin inhibition.

The other criteria of calpastatin-calpain interaction is that they must be physically in the same location. From the limited immunolocalisation data available this apparently is the case in muscle cells (31), although the immunofluorescence was not very clear.

Proteolysis of calpastatin may be a critical factor in the modification of the inhibitory capacity of the protein. Extraction procedures for calpastatin have shown how susceptible it is (51,67). The evidence that calpastatin is a suicide substrate (65) along with its proteolytic susceptibility may account for the high levels of calpastatin expression in cells, generally being much greater than calpain activity. But this could be a means of overcoming calpastatin inhibition in a localised area of a cell during high calpain activity.

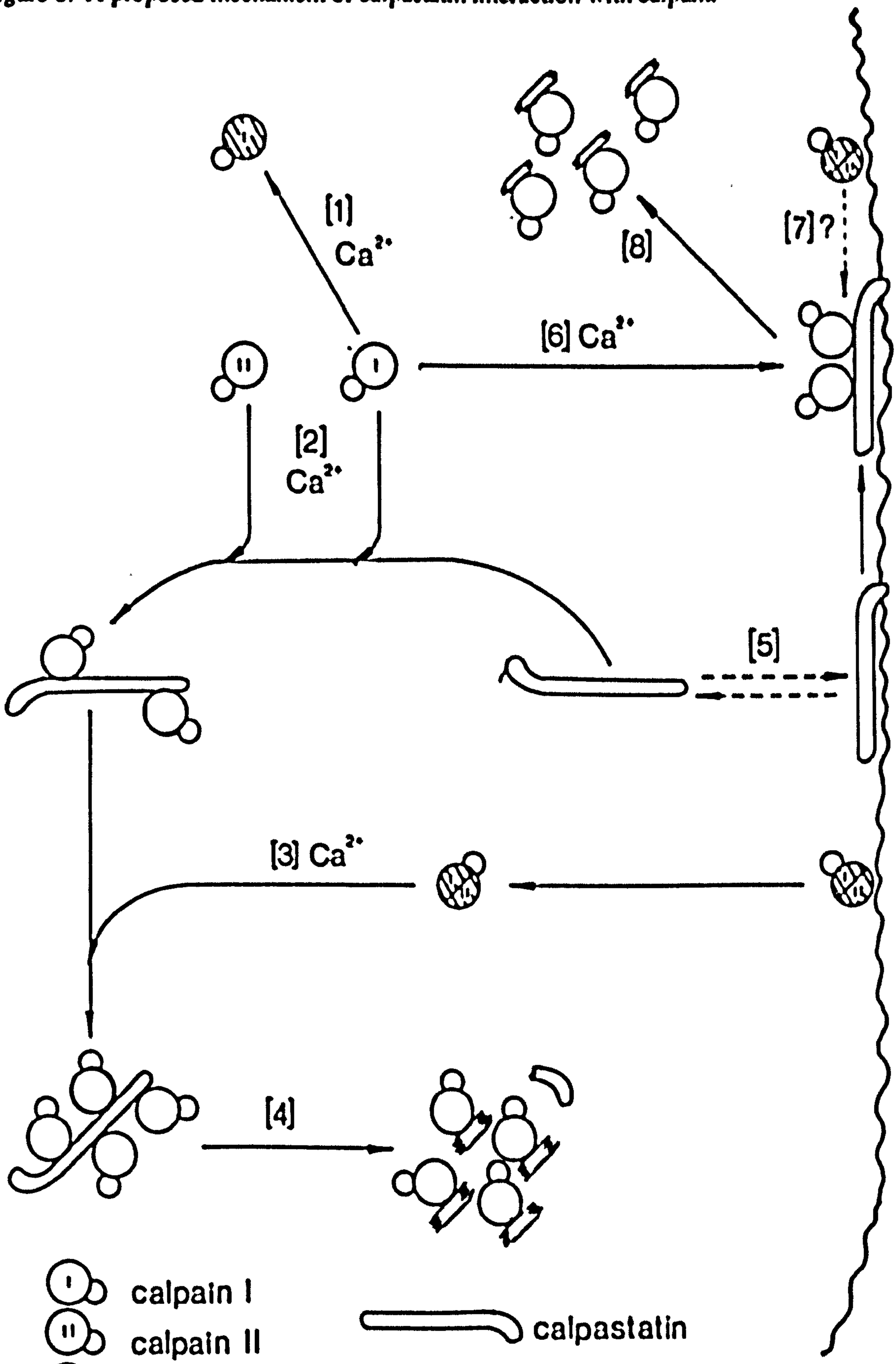
2.2.3. A Proposed Mechanism for Interaction of Calpastatin with Calpain.

Figure 6 shows an illustration of the possible mechanism of calpastatin interaction with calpain, and thereby the possible means by which calpain may be active in vivo even in the presence of calpastatin.

Unautolysed calpain I could be active in the cytosol when calcium concentrations reach the lower end of its activity requirement, at approximately 10μM [1]. However if local calcium concentrations do increase further, above 40μM, this would cause the association of calpastatin to calpain [2], for both isoforms.

In order for calpain I and II to be active at physiological Ca^{2+} concentration association with the membrane is required, as described in section 2.1.2.. The mechanism of interaction with the plasma membrane and subsequent autolysis may be different for each isoform. The autolysed cytosolic calpains released from the

Figure 6: A proposed mechanism of calpastatin interaction with calpain.



membrane are thought to be inhibited by calpastatin without being active [3]. Although calpain I may be active in the cytosol calpain II is not, but it may be activated in this environment by the association with specific activators. These apparently prevent calpastatin inhibition but have no effect on calpain I (section 2.1.2.).

Calpastatin can inhibit more than one molecule of calpain [4] but will become degraded as it is thought to be a suicide inhibitor/substrate. It appears that removal of calcium from the environment of the calpain-calpastatin complex releases calpastatin fragments some of which still retain inhibitory activity (65). The implication of this in vitro observation in vivo is not known.

A small proportion of the calpastatin is associated with the membrane through the L domain [5]. This membrane-bound calpastatin is believed to bind calpain in the presence of calcium [6] and could act in competition with the membrane binding/substrate proteins which are involved in calpain I association to the membrane. The exact calcium concentration at which this takes place is not known nor whether active or inactive membrane associated calpain will also bind to the inhibitor located on the membrane [7]. Association of several calpain proteins to the membrane bound calpastatin probably leads to its dissociation from the membrane following the cleavage of domain L from the inhibitor and further proteolysis of the calpastatin in the cytosol [8].

The model for this calpain-calpastatin interaction and its effect on calpain mediated proteolysis is based on the observations reported in this review but should be considered as being speculative.

2.2.4. Summary

Calpastatin is a specific endogenous inhibitor for calpain. Its interaction with calpain is calcium dependent and the concentration at which the calpastatin-calpain complex is formed is different for each isoform. From in vitro observations it appears that the binding of calpain to calpastatin occurs close to or below the calcium concentration required for activation. The calcium required for the formation of the calpain-calpastatin complex is believed to associate with calpain. The enzyme probably

undergoes a conformational change in order to bind to calpastatin. These regulatory mechanisms suggest that calpain mediated proteolysis is under tight regulation. However, in vivo the inhibitory effects of calpastatin may be affected by other factors such as interaction with membranes and calpain activators which may allow the loosening of the inhibition constraints.

2.3.0. Substrates for Calpain and its Involvement in Protein Turnover.

Calpain substrates can be broadly categorised into groups of;

i) Myofibrillar proteins

ii) Membrane proteins

iii) Cytoskeletal proteins

iv) Receptor proteins

v) Enzymes.

Many of the substrates for calpain do belong to more than one of these groups. The first category to be considered is the role of calpain in the degradation of myofibrillar proteins.

2.3.1. Calpain's Role in Skeletal Muscle Myofibrillar Protein Degradation.

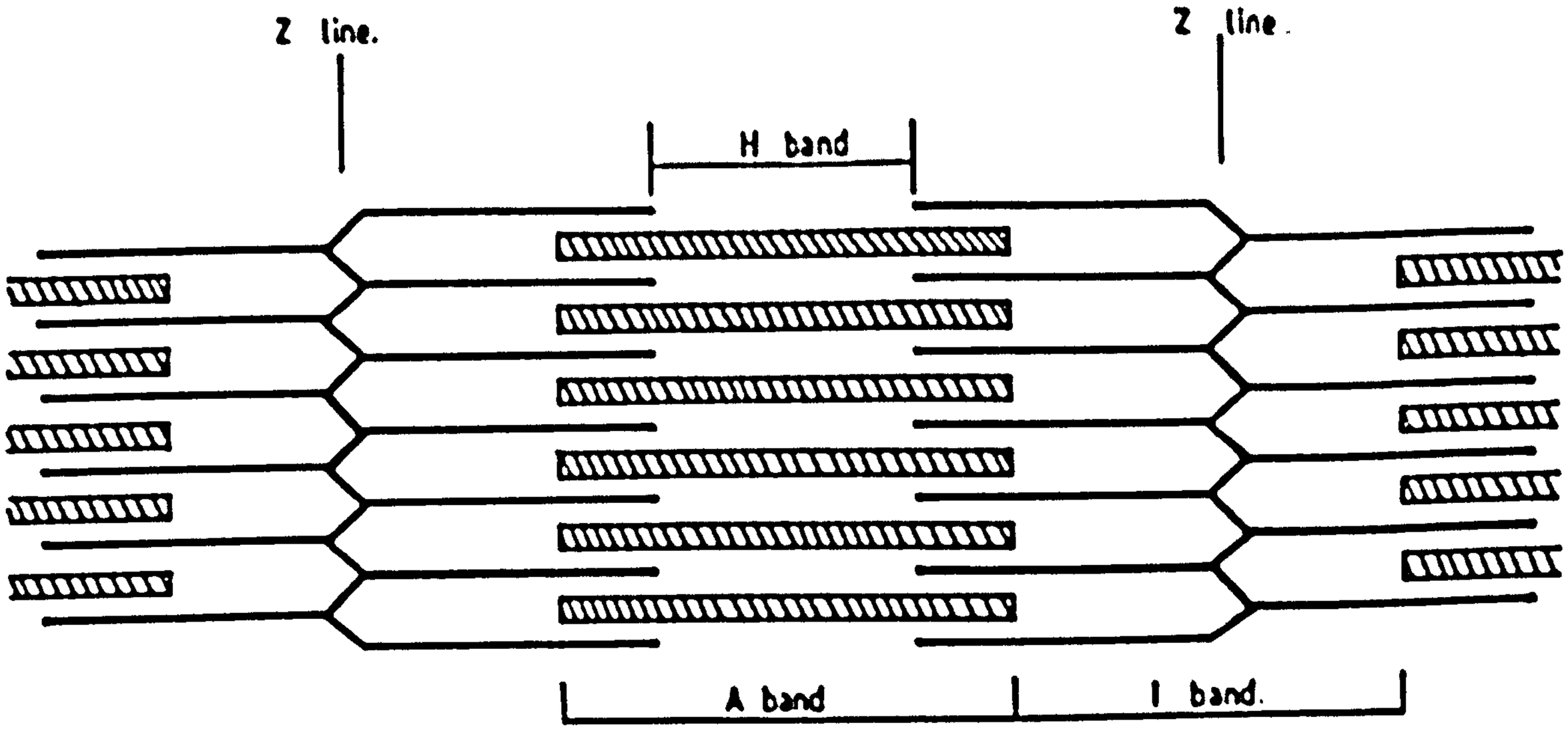
Myofibrillar protein substrates.

One of the first effects of calpain proteolytic action was indicated in skeletal muscle protein degradation (3). In isolated myofibrils incubated with calpain II at mM calcium concentrations there was removal of the Z-line whose position is shown in Figure 7. The elimination of the Z-line releases proteins associated with it including α-actinin, which is not a substrate for calpain, and zeelins that are (68,69). The specific substrates within the Z-lines which are degraded by calpain and lead to its dissolution are not known. Most of the initial studies were carried out using calpain II, however calpain I is also capable of removing Z-lines from myofibrils (70).

Various purified myofibrillar proteins have been shown to be calpain substrates including troponin, tropomyosin and C-protein (71). Isolated myosin is degraded but as with most calpain substrates it is specifically cleaved in certain positions (72). The large cytoskeletal proteins titin and nebulin are also proteolytically fragmented (73). These are thought to be involved in the maintenance of sarcomere structure, the large protein titin spanning half its length from Z-line to M-line.

Although these myofibrillar proteins are substrates, such studies do not conclusively prove that they are degraded in vivo. The repeated observations that calpain

Figure 7: Schematic diagram of muscle myofibril sarcomere structure.



Thick filament

Thin filament

degrades Z-lines in myofibrils does suggest its involvement in muscle turnover. By removing Z-lines and degrading titin calpain could be a means of 'loosening' the myofibrillar structure thereby allowing other proteinases to carry out the major proteolytic events involved in protein turnover of muscle. Calpain has been immunolocalised at the Z-lines and the surrounding I-bands (74,75), but in other studies it has been seen distributed throughout the cytosol (31,76) and at the plasma membrane (77). Both calpain I and II have been associated with the specific degradation of numerous membrane proteins to be discussed in section 2.3.2..

Calpain activity in skeletal muscle protein turnover.

It is difficult to prove that calpain is involved in the cycle of protein turnover in skeletal muscle as there are no absolutely specific inhibitors to calpain, apart from the possible exception of calpastatin which will not penetrate membranes and furthermore is susceptible to proteolysis. Most of the inhibitors used in the early experiments to determine the role of calpain are general cysteine proteinase inhibitors. Although the specificity of some of the more recent synthetic inhibitors is quiet high the homology of the cysteine proteinases around their catalytic domain makes it difficult to obtain absolute specificity toward calpain (78). This also suggests that in order for calpastatin to be specific toward calpain there is likely to be some other interaction of the inhibitor with the enzyme as it is recognized that the synthetic inhibitors associate with the active site of the enzyme.

An example of use of a cysteine proteinase inhibitor to implicate calpains in muscle protein turnover was described by Badalamente et al (79) who used the inhibitor leupeptin to facilitate the recovery of denervated muscle after nerve lesion and subsequent repair. They suggested that it was by inhibition of calpain in the tissue that recovery was enhanced. However leupeptin is known to be a general cysteine proteinase inhibitor (78), so that such results do not exclude the role of calpain in muscle degradation but they do not confirm it unequivocally.

Although calpain is likely to be implicated in muscle protein turnover there are few definitive results proving its direct involvement. There have been several studies on

isolated skeletal muscle that relate the calcium activated proteolytic systems in muscle protein turnover to other protein degradation mechanisms. In vitro protein turnover experiments are difficult to interpret as they use cysteine proteinase inhibitors and/or calcium ionophores to indicate that calpains are part of the increased calcium induced proteolysis (80-82). While Rodemann et al (80) reached the conclusion that calpain was not involved in general proteolysis, Zeman et al (81) and Furuno et al (82) drew the opposite conclusion. The differences in the studies are usually caused by lack of consènsus on the specificity of the inhibitors and whether they reach all the sites of calpain activity. Overall the use of methods to increase free calcium leads to the elevation of proteolysis, and the employment of calpain/cysteine proteinase inhibitors tends to prevent this calcium induced increase in protein degradation (81,82). Such evidence supports the likelihood of calpain being involved at some stage in muscle protein turnover.

Augmented calpain activity associated with skeletal muscle atrophy.

An increase in calpain activity has been observed where there are states of elevated degradation of muscle proteins. This can considered to be further evidence that calpain may be involved in the turnover of myofibrillar proteins. Induced atrophy caused by vitamin E deficiency resulted in an increase of 3.6 times the control levels of the total calpain activity in rabbit skeletal muscle (83). Ultrastructural observations of changes in the myofibrils showed a decrease in Z-line density.

Muscular dystrophy also results in changes in the activity of the components of the calpain system (84-88). There are few studies where all the components of the calpain system have been separated and their activity assessed. When all the studies are considered they give conflicting results. In human Duchenne muscular dystrophy an increase in calpain I and II was observed with no change in calpastatin activity (85). However in dystrophic hamster muscle there was an increase in both calpain isoforms and calpastatin activities at four weeks old, but at ten weeks there was no change in calpain I and II but an increase in calpastatin activity (86).

Increases in calpastatin activity along with calpain II were seen in mice and hamsters (86,87). The difference in the observations may be due to the age at which the assessment of activity was made, especially in the genetic models of muscular dystrophy in laboratory animals. Initial events in these dystrophic animals involve degeneration of the fibres which is closely followed by on going regeneration by activation of satellite cells. The two processes are taking place simultaneously. In mdx mice this counterbalancing effect of increased muscle protein synthesis results in animals that are never severely weakened and grow larger and stronger than the wild type animals (89). Such events may require an initial increase in calpain mediated proteolysis, but subsequently, as the regeneration becomes more prominent, inhibition of calpain by calpastatin increases.

There is an increase in free calcium in skeletal muscle cells of dystrophic (mdx) mice which could activate calpain (90). The involvement of other proteolytic systems in muscular dystrophy is not discounted but the genetic disease does appear to entail enhanced calpain activity which could be further stimulated by increases in intracellular free calcium. The degradation pattern of muscle proteins is similar to that observed when isolated myofibrils are digested with calpain (3), and this, along with changes in the calpain activity, does suggest an involvement in muscle protein turnover in this myopathy.

Further evidence of calpain-induced proteolysis in protein degradation was observed in denervated muscle undergoing atrophy, when calpain levels were increased and calpastatin levels were decreased, leading to an increase in proteolytic potential in the calpain system (91).

2.3.2. Other Substrates for Calpain.

Calpain susceptibility occurs in a wide range of substrates but as with the degradation of muscle proteins the substrates of calpain mediated proteolysis can be identified as specific proteins.

Membrane associated calpain substrates.

As described above calpains are known to associate with membranes via phospholipid interaction and possibly via specific substrate or membrane protein binding association. The enzyme has been shown to fragment some of the proteins lining the cytosolic side of the membrane; these include spectrin (92), band 3 protein (92), fodrins (93,94) and band 4.1 proteins (92). Calpain has been implicated in the rearrangement of cell membrane structural proteins (49). There is a variation in the distribution of calpain II between nonfusing myoblasts and their fusing counterparts (95). In cells that have the capability of fusing, calpain II is predominantly associated with the plasma membrane, whilst in nonfusing cells it is mainly cytosolic. The induced fusion of rat erythrocytes in the presence of calcium can be inhibited in the presence of EGTA and cysteine proteinase inhibitors (49). Calpain could act by removing the submembrane proteins to expose the lipid bilayer which would be more susceptible to fusing with similar membranes. There is evidence of changes in the activity of calpain during the formation of skeletal muscle myotubes. Kaur and Sanwal (96) observed an increase in total calpain activity in the supernatant of centrifuged myotube homogenates, and also described the decrease of a endogenous inhibitor to calpain, which they failed to identify but was probably calpastatin. This was expressed before fusion and extracts from the prefusion cells would inhibit the total calpain activity seen in the fusing myoblasts. Kaur and Sanwal further suggested that this inhibitor could regulate the enzyme activity by being destroyed in myoblast fusion without the need for changes in the gene expression of the enzyme, although the latter cannot be ruled out.

Cytoskeletal calpain substrates.

Several cytoskeletal proteins have been identified as substrates for calpain including the intermediate filament proteins vimentin and desmin (97), the microtubule-associated protein Tau which has been implicated in Alzheimer's disease (98), and the microtubule protein tubulin (99,100). Calpain II has been found associated with cell adhesion plaques, that are implicated in the cytoskeleton and cell motility, which contain a substrate for calpain, talin in their structure (101). Calpain digestion could possibly lead to the dissolution of the plaque and the rearrangement of the cytoskeleton.

Calpain II has been connected with the structural alterations seen in mitosis (102). Microinjection of calpain II initiated the onset of mitosis, and calpain immunolocalization moved to the mitotic chromosomes from being associated with the membrane. From her observations Schollmeyer (102) suggested that calpain II was active at the cytosolic calcium concentration which is well below that required in vitro. The study also indicated that calpain migrated within the cell during the course of mitosis. This may be a means by which the enzyme could overcome the effects of calpastatin.

All these studies have shown that calpain may be responsible for alterations in the cytoskeletal structure of cells. In order for a cell to become capable of fusing or undergo cytoskeletal rearrangement proteolysis has to be carried out to remove cellular structure already present. As calpain is specific to many of the cytoskeletal proteins, under the influence of the inhibitory control and activated by changes in the calcium ions limited proteolytic degradation may be achieved. Other evidence which points to this role is calpain's association with specific areas of cytoskeletal anchoring and the large scale rearrangement seen in mitosis.

Calpain mediated proteolysis of receptors.

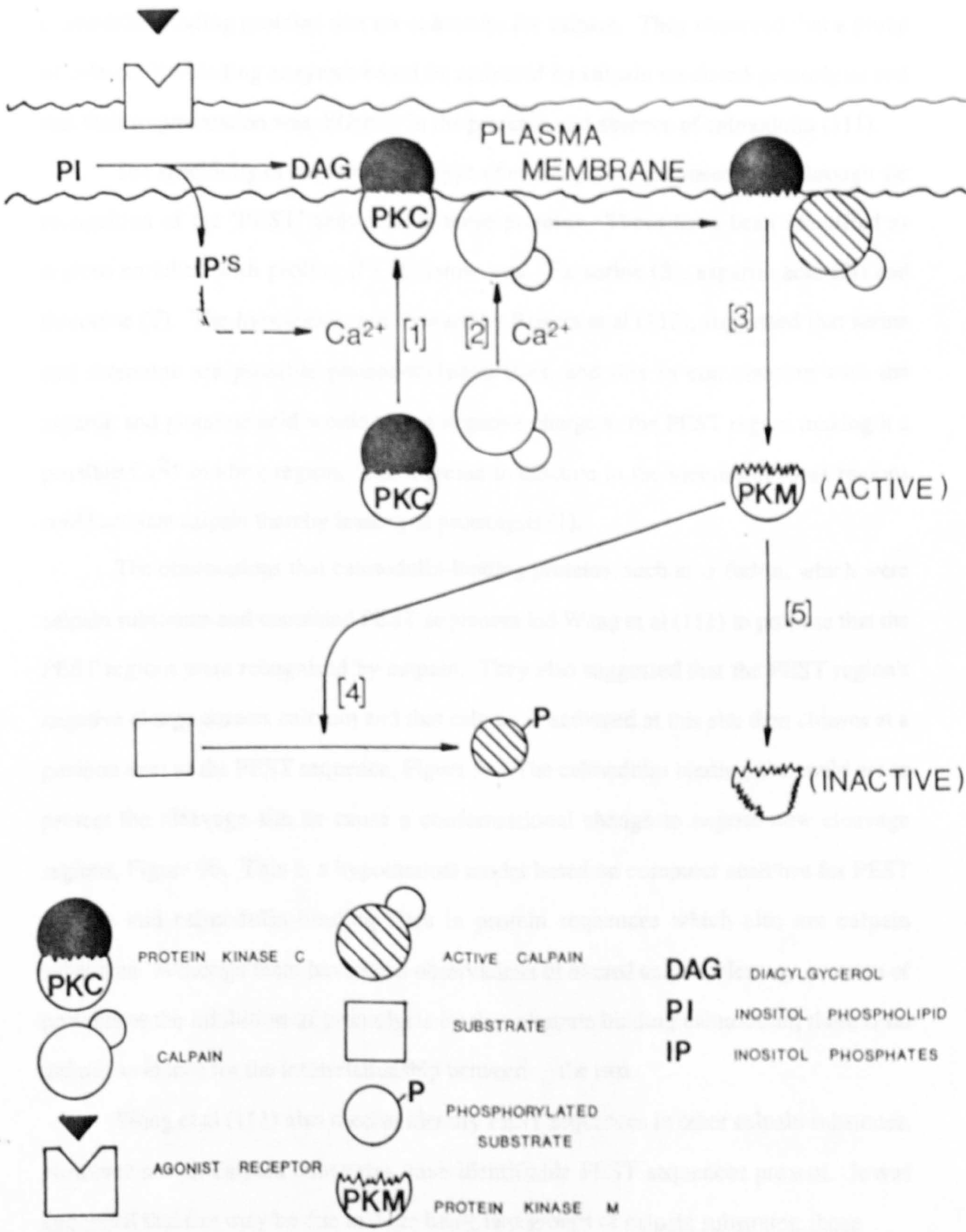
Several receptors are also sensitive to proteolysis by calpain, including the membrane associated receptors for EGF (103) and the α-adrenergic agonists (104), as well as the cytosolic receptors for progesterone (105) and oestradiol (106). Again proteolytic fragmentation is specific to key sites and not random which may suggest a possible involvement in down regulation or translocation.

Proteolysis of protein kinase C: an example of calpain action on enzymes.

The involvement of calpain in an event which can be interpreted either as enzyme activation or down regulation is the proteolytic fragmentation of protein kinase C (107,108). The cleavage of the membrane associated protein kinase C produces a fragment that is active as a kinase but does not require calcium or phospholipids for activity, unlike native protein kinase C, the regulatory domain having been removed from the kinase domain (107). There is more than one isoform of protein kinase C (109): three of the isoforms, type I (gamma), type II (beta I and beta II) and type III (alpha) have been shown to be proteolytically cleaved by calpain I and II (110). Calpain I is the more effective of the two enzymes: it cleaves the various kinases at different rates and preferentially the diacylglycerol activated form in the same region of the enzyme. The catalytic fragment, the kinase domain, is active and has the potential to phosphorylate substrates which are not exposed to the membrane-bound active protein kinase C (107,108). Calpain mediated proteolysis may be the first step in the activation of a cytosolic kinase which phosphorylates a different spectrum of substrates to cAMP dependent protein kinase, but also effects metabolic events in the same manner. However, an alternative view is that the cleavage of protein kinase C may be the first step in the complete degradation of the enzyme i.e. 'down-regulation'.

A proposed mechanism is outlined in Figure 8. The stimulation of diacylglycerol production with the coincident release of inositol phosphates by a receptor-mediated event activates the membrane associated protein kinase C in the presence of calcium ions [1]. This membrane bound active protein kinase C is then open to cleavage by calpain which probably associates with the membrane due to elevation of the calcium concentration [2]. The resulting proteolysis of protein kinase C [3] produces a membrane-independent kinase which is capable of phosphorylating cytosolic substrates [4], but is also further degraded to an inactive form [5].

Figure 8: A model for the calpain-mediated proteolytic activation of protein kinase C (107).



2.3.3. A Proposed Mechanism by which Calpain Mediated Proteolysis Selectively and Specifically Cleaves Substrates.

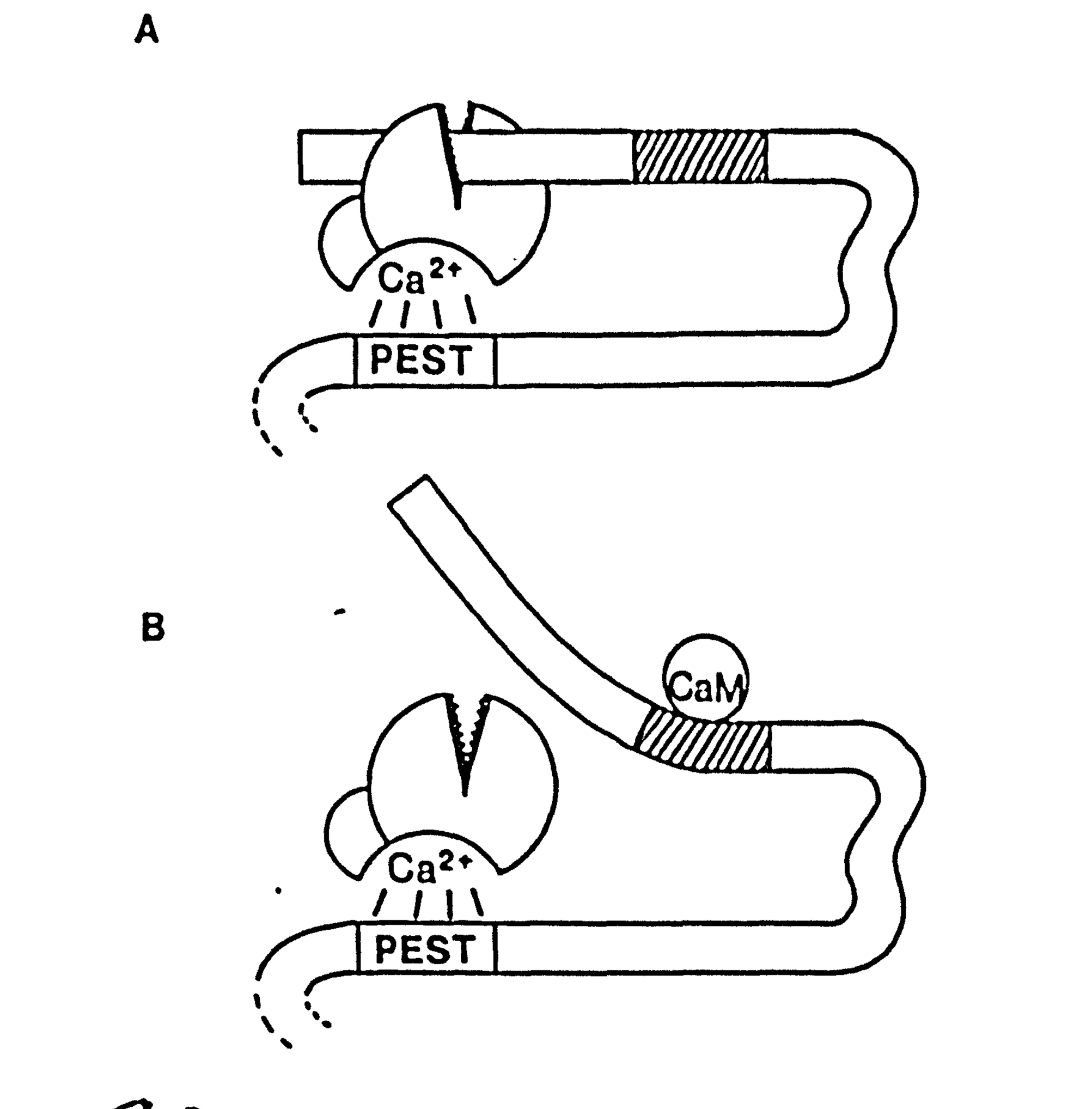
A model for the selective proteolytic action of calpain on substrates has been proposed by Wang et al (111), based on their observations of calcium sensitive calmodulin-binding proteins that are substrates for calpain. They observed that a group of calmodulin-binding enzymes could be activated by calpain mediated-proteolysis and that their fragmentation was different in the presence and absence of calmodulin (111).

The specificity of calpain in this type of cleavage was proposed to act through the recognition of the 'PEST' sequence in these proteins. These have been identified as regions enriched with proline (P), glutamic acid (E), serine (S), aspartic acid (D) and threonine (T). The hypothesis, put forward by Rogers et al (112), suggested that serine and threonine are possible phosphorylation sites, and this in combination with the aspartic and glutamic acid would give a negative charge to the PEST region making it a possible Ca^{2+} binding region. The increase in calcium in the vicinity of these regions could activate calpain thereby leading to proteolysis (1).

The observations that calmodulin-binding proteins, such as α-fodrin, which were calpain substrates and contained PEST sequences led Wang et al (111) to propose that the PEST regions were recognised by calpain. They also suggested that the PEST region's negative charge attracts calcium and that calpain is activated at this site then cleaves at a position next to the PEST sequence, Figure 9a. The calmodulin binding site could act to protect the cleavage site or cause a conformational change to expose new cleavage regions, Figure 9b. This is a hypothetical model based on computer searches for PEST regions and calmodulin-binding sites in protein sequences which also are calpain substrates. Although there have been observations of altered calpain cleavage patterns of proteins or the inhibition of proteolysis by the substrate binding calmodulin, there is no definite evidence for the interrelationship between the two.

Wang et al (111) also tried to identify PEST sequences in other calpain substrates. However not all calpain substrates have identifiable PEST sequences present. It was suggested that this may be due to there being two groups of calpain substrates; those

Figure 9: A model for PEST sequence mediated calpain proteolytic action (a) and its modification by calmodulin interaction with the substrate (b) (111).



degraded by the PEST recognition mechanism and those which are subjected to proteolysis by some other means.

PEST directed protein degradation would tend to give specific fragments. Similar fragmentation of proteins by calpain proteolysis does suggest that calpain recognition of sites is under the influence of some other factor, like the PEST sequence, rather than the random interaction of the cysteine proteinase active site with the primary amino acid sequence. The PEST hypothesis gives an added dimension of sensitivity in calpain proteolysis as well as proposing a means of increasing calcium concentrations in the immediate vicinity of the substrate to be cleaved.

2.3.4. Summary

Although calpain does act on muscle myofibrillar structure, it is also capable of cleaving a range of substrates as described above. The proteolysis appears to produce specific fragmentation of proteins. It is possible that calpain is involved in a more regulatory capacity than a general proteolytic enzyme which may have indirect implications on protein turnover. Calpain appears to have the capacity to irreversibly activate enzymes such as protein kinase C which is proteolytically cleaved and released into another dimension where it is active. This altered kinase activity could have implications on the metabolic status of the cell as it is known to affect the rate and pattern of gene expression.

2.4.0. The β-Adrenergic Agonists and their Effects on Skeletal Muscle Growth with Relevance to Calpain and Calpastatin Activity.

In the previous section the possible role of the calpain system in muscle protein turnover was examined. From the observations of a number of other groups the calpains do act on specific myofibrillar proteins, as well as proteolytically controlling the activity of enzymes and receptors involved in metabolic regulation. In order for muscle growth to take place there has to be net protein deposition. At its simplest this can be achieved by increased protein synthesis and/or decreased degradation. If calpain is involved in the process of muscle protein turnover a decrease in its activity could be one of the means by which increased muscle growth could be achieved. This could conceivably be through a decrease in activity of calpain by a reduction in the quantity of the enzyme or by increased inhibition of the activity already present by agents such as calpastatin.

The effect of changing growth rate on the activity of calpain and calpastatin has been examined in our laboratories by Ballard et al (113). In these experiments, chickens were grown at different rates by varying the level of dietary protein. The rate of growth was significantly increased in the presence of a greater proportion of protein in their diet. When the activities of calpain and calpastatin were assessed there was no statistically significant change in either of them.

A further growth study in our laboratories by Higgins et al (114), on lambs, examined the components of the calpain system, calpain I, II and calpastatin, in animals on two diets which gave different growth rates. The Longissimus dorsi (L.dorsi) muscle was examined. It was of lower weight and cross sectional area in the restricted intake group but showed no change in the calpain system activity. However when lambs were fed at the higher intake level plus the β-agonist clenbuterol (a gift from Bochringer Ingleheim Vetmedica Gmbh), at 2ppm for six weeks, there was a significant increase in the weight and cross-sectional area of the L.dorsi along with a significant change in the activities of all three components of the calpain system relative to the controls on the higher dietary intake. Calpain II and calpastatin increased in activity (units/kg) by 99%

and 130% respectively of their control values whilst calpain I activity fell by 14% (Table 2.A.).

The effects of β-agonists on skeletal muscle.

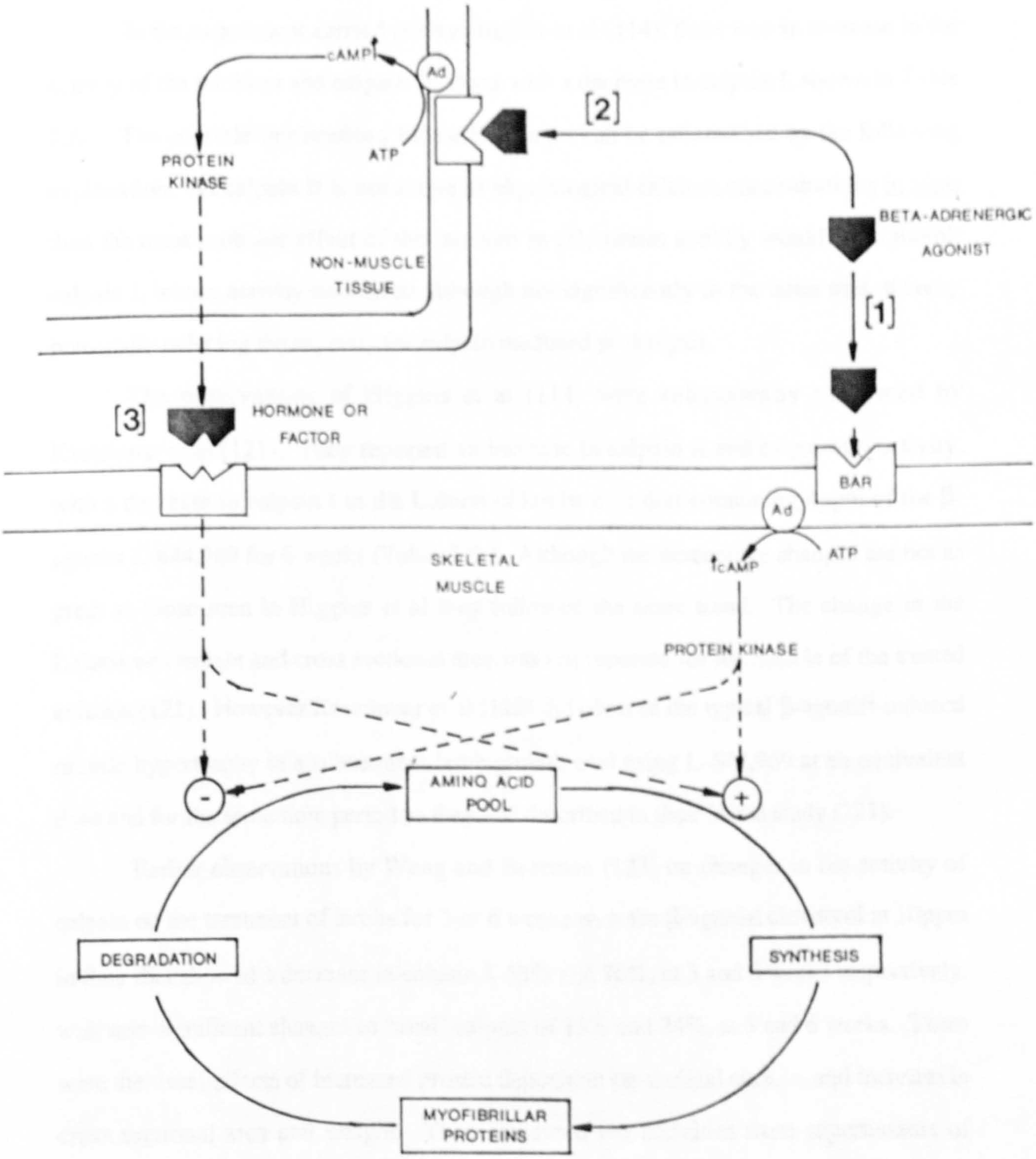
The β-agonists are known to act as repartitioning agents, that is they increase skeletal muscle growth whilst decreasing body fat (115). Muscle growth is achieved through hypertrophy and the effect appears to be restricted to skeletal muscle (115). The mechanism of action by β-agonists is believed to be through decreased protein breakdown (116-118). However some studies have suggested increased synthesis may also be responsible for changes in net deposition (119,120) as well as the decreased degradation. The observations may be partially dependent on the dose, the mode by which it was given (injection or orally), after what period the synthetic rate was assessed and the type of agonist used.

The action of β-agonists appears to be directly through the β-receptor, as stimulation by injection of the β-agonist clenbuterol intraperitoneally into rats led to an increase in muscle cAMP (120). These effects as well as those on growth were abolished by administration by injection of propranolol (a β-antagonist) before the dose of clenbuterol.

Figure 10 shows the possible means by which β-adrenergic agonists could induce skeletal muscle hypertrophy. The evidence points to β-agonists acting directly through β-receptors causing a rise in intracellular cAMP via adenylate cyclase stimulation [1] with subsequent activation of cAMP dependent protein kinase (protein kinase A), thus having an effect on muscle protein turnover by protein phosphorylation events. Alternatively β-agonists could act in a paracrine manner on nonmuscle cells [2] stimulating the release of hormones or other growth factors [3], which activate or regulate growth independently or in conjunction with the direct effects of β-agonists on muscle.

As the β-agonist effect on increased protein deposition in skeletal muscle appears to be specific then the post-receptor events after stimulation must be different in muscle to other tissues. Protein degradation appears to be involved in the response to β-agonists as there is a recognized decrease in treated animals. If this is the case then there must be a

Figure 10: A possible mechanism for the β-adrenergic agonist induced skeletal muscle hypertrophy (115).



BAR BETA-ADRENERGIC RECEPTOR

Ad ADENYLATE CYCLASE

decrease in the proteolytic activity mediated by a reduction of the expression of the proteinases involved or an increase in their inhibition.

The effects of β-agonists on the skeletal muscle calpain system.

In the experiment carried out by Higgins et al (114), there was an increase in the activity of the inhibitor and calpain II, along with a decrease in calpain I, shown in Table 2.A... The possible implications for such changes can be rationalised by the following explanation. As calpain II is not active at physiological calcium concentrations in vitro then the most probable effect of the increase in calpastatin activity would be to inhibit calpain I, whose activity decreased although not significantly in the lamb trial, thereby potentially reducing the capacity for calpain mediated proteolysis.

The observations of Higgins et al (114) were subsequently confirmed by Kretchmar et al (121). They reported an increase in calpain II and calpastatin activity, with a decrease in calpain I in the L.dorsi of lambs on a diet containing 4ppm of the β-agonist L-644,969 for 6 weeks (Table 2.B.). Although the percentage changes are not as great as those seen in Higgins et al they followed the same trend. The change in the L.dorsi wet weight and cross sectional area was not reported for the muscle of the treated animals (121). However Kretchmar et al (122) did observe the typical β-agonist-induced muscle hypertrophy in a subsequent lamb growth trial using L-644,969 at an equivalent dose and for the same time period as they had described in their initial study (121).

Earlier observations by Wang and Beerman (123) on changes in the activity of calpain on the treatment of lambs for 3 or 6 weeks with the β-agonist cimaterol at 10ppm in their diet showed a decrease in calpain I, 55% and 70%, at 3 and 6 weeks respectively, with non-significant changes in 'total' calpain of 13% and 24%, at 3 and 6 weeks. There were the usual effects of increased protein deposition on skeletal muscle, and increase in cross sectional area and weight. They measured the activities from supernatants of centrifuged crude homogenates of L.dorsi using different calcium concentrations in the assays to distinguish between the calpain I and 'total' calpain activities, 250μM and 5mM calcium respectively. These measurements are complicated by interactions of the inhibitor with both isoforms of calpain and autolysis of the enzyme. Such anomalies are

Table 2: Comparison of the effects of β-agonists on the activities of calpain I, II and calpastatin in skeletal muscle. All activity determinations were carried out on L.dorsi muscle, except C where the assays were on a combined sample of biceps femoris, gastrocemius and semimembranosus.

		control	β-agonist	% change	signif.
A.		(n=6)	(n=6)		
		(units/kg muscle)			
Lamb trial	Calpain I	570	490	-14	NS
Higgins	Calpain II	1250	2880	130	$p< 0.05$
et al,(114)	Calpastatin	2740	5440	99	$p< 0.05$
B.		(n=11)	(n=11)		
		(units/kg muscle[a])			
Lamb trial	Calpain I	550	500	-10	$p< 0.05$
Kretchmar	Calpain II	650	950	41	$p< 0.05$
et al,(121)	Calpastatin	1250	2200	74	$p< 0.05$
C.		(n=6)	(n=6)		
		(units/g protein)			
Rabbit trial	Calpain I	10.3	4.3	-58	$p< 0.05$
Forsberg	Calpain II	48.8	21.0	-56	$p< 0.05$
et al,(124)	Calpastatin	290.0	138.0	-52	$p< 0.05$

NS = non-significance $p> 0.05$

[a] These values were expressed graphically by Kretchmar et al (124) using a different definition of units so the values shown are approximations

reduced by the use of ion-exchange chromatography to separate the activities so that they are assessed individually, as in Higgins et al (114) and Kretchmar et al (121). The study does give an indication of the overall effect of treatment of β-agonists on the skeletal muscle calpain system. Wang and Beerman (123) observed an apparent decrease in the calpain mediated proteolytic potential at low calcium concentrations, this could have been caused by an increase in calpastatin activity or a decrease in calpain activity at this calcium concentration (250μM), or both as seen in the observations of Higgins et al (114) and Kretchmar et al (121).

Forsberg et al (124) studied the effect of the β-agonist cimaterol on rabbits and recorded different results to the ones seen in lambs(114,121). In rabbits fed cimaterol at 10 ppm for 5 weeks there was a decrease in the activity of calpain I, II and calpastatin (Table 2.C.). The assays were carried out on a mixed group of muscles which showed a significant increase in weight in response to β-agonist treatment. However it is not known whether the expression of the calpain system components is the same in different muscles let alone the response of the calpain system in different types of muscle to β-agonist. The difference to the observations discussed above may have been due to the species used and possibly the extraction procedure. Calpastatin is susceptible to proteolysis (51,67), and in Forsberg et al methodology they dialysed the supernatant of the centrifuged crude homogenate for 24 hours before separation of the components of the calpain system on ion-exchange chromatography. This may have allowed proteolytic cleavage of the calpastatin to take place. In support of this they reported that calpastatin activity did not elute as a discrete peak and 'small quantities of additional calpastatin activity' eluted following the calpain I on DEAE Sephacel columns. This suggests that calpastatin activity may have been present in the calpain I peak thereby reducing the true activity. The spread of inhibitor elution also implies degradation of the large calpastatin molecule which could lead to its reduced activity. The reduction in calpain II activity where previous reports have shown an elevation may be caused by inhibition by proteolytic fragments of calpastatin which has been observed in other studies (65), described in section 2.2.3..

Beta-agonist mediated effects on cathepsins.

In addition to assessing the activities of the calpain system, both Forsberg et al (124) and Kretchmar et al (121) examined the cathepsins. The majority of cathepsins are cysteine proteinases whilst cathepsins D and E are aspartic proteinases. Forsberg et al (124) found no change in cathepsin B and D activity, whilst Kretchmer et al (121) found a decrease in cathepsin B specific activity and an increase in cathepsin H. McElligott et al (125) reported that clenbuterol treatment of rats for a week caused no change in cathepsin B and D activity in the EDL and gastrocnemius muscles whilst soleus muscle showed an increase. At two weeks there was decreased activity of cathepsin B in the muscles which had shown no change at one week, with the soleus response the same, namely an increase (126). These proteinases are compartmentalized in lysosomes so that their activity is modulated. Also there are inhibitors of the cathepsin B and H which could influence the activity of the enzymes assayed if they are not removed, as in the calpain system.

Unlike some of the cathepsins, which require low pH for activity and are largely compartmentalized in the acidic environment of the lysosomes (127), calpains may have an important regulatory role in protein turnover. From the observations with the effect of β-agonists the activities of calpains are altered and from the influence of the inhibitor there is a probable decrease in the proteolytic capacity of the system.

The effects of β-agonists on skeletal muscle atrophy.

The reduction of proteolysis by the use of β-agonists has been investigated in studies on metabolic states where there is known muscle atrophy. A reduction of the severity of muscle atrophy induced by denervation was achieved by the use of the β-agonist clenbuterol, described by Zeman et al (128) in rats. Denervation of skeletal muscle has been shown to increase calpain activity whilst decreasing calpastatin (91). The effect of β-agonist treatment on the calpain system of skeletal muscle may be partially responsible for the reversal of the atrophy in denervated muscles by suppressing calpain mediated proteolysis.

The calpain system has been implicated in the atrophy seen in muscular dystrophy (84-88), as described in section 2.3.1.. The effect of clenbuterol was studied on dystrophic (mdx) mice (129). Treatment with the β-agonist resulted in the recovery of body composition toward that of the controls. The mdx mice have higher body fat, lower protein content and reduced muscle mass. Clenbuterol treatment increased the muscle mass to a level greater than that for the treated normal mice. As with denervation atrophy it could be speculated that the β-agonists affected the activity of calpain by increasing the inhibitory capacity of calpastatin thereby decreasing protein degradation. However as described earlier there are problems in studying mdx mice as a model of human Duchene muscular dystrophy. The mdx animals may not have been in a state of true atrophy as regeneration processes are also seen in their skeletal muscles. This further complicates the interpretation of the observations with relevance to the calpain system.

In both these cases of altered proteolytic status the exact role of calpain is not known, therefore the case for β-agonists mediating their effect by inhibiting calpain action is speculation. However there is strong evidence that calpain is involved in the muscle hypertrophy induced by β-agonists.

2.4.1. Summary

Beta-agonist induced skeletal muscle hypertrophy appears to be brought about by reduced protein degradation and as well as a possible increase in synthesis resulting in protein accretion. Examination of the calpain system in treated animals indicates there is probably inhibition of its proteolytic potential brought about by an increase in calpastatin activity. The lack of changes in the activities of the calpain system components seen when growth is regulated by diet is probably due to the change in protein turnover not being through the same mechanisms as β-agonist induced hypertrophy, namely a significant reduction in protein degradation

Use of β-agonists in states of increased skeletal muscle atrophy, where calpain activity has been implicated, have shown that the agonists reduce skeletal muscle wasting. This may be mediated through diminished calpain activity.

2.5.0. The Effect of Receptor-Induced cAMP on the Regulation of Enzyme Activity.

As described in the previous section calpain and calpastatin activity is altered in skeletal muscle by the effects of β-agonists. Apparently the β-agonists do not act directly on calpain by intermolecular interaction as there was no change in activity when cimaterol was added to calpain (123). Accordingly, one or other of the known cAMP-mediated stimulatory mechanism may be called into play.

2.5.1. Possible Phosphorylation Induced Changes in the Activity of the Calpain System Mediated by the Secondary Messenger cAMP.

In the skeletal muscle growth response β-adrenergic agonists presumably act through post β-receptor-mediated events similar to those in other tissues. The β-agonists bind to β-receptors which then interact with G-proteins and in turn these stimulate adenylate cyclase leading to an increase in cAMP levels, subsequently activating cAMP-dependent protein kinase. The kinase activates by phosphorylation enzymes which are involved in generally degradative metabolic processes, for example the enzyme activation cascade leading to glycogenolysis. This type of regulation may have effects on the calpain system by mediating reversible changes in activity.

Murachi's group have described the phosphorylation of calpastatin (130). Transfection of the T-cells gives increased expression of calpastatin (130). In these cells labelling of calpastatin in vivo with [^{32}P]-orthophosphate in the presence and absence of phorbol esters was detected. Phosphorylated calpastatin was subsequently shown associated with the cell membrane whilst nonphosphorylated inhibitor was located in the cytosol. There was no apparent change in the proportion of phosphorylated calpastatin associated with the membrane when phosphorylation took place in the presence or absence of phorbol ester.

The significance of this phosphorylation in terms of activity is not known but Murachi suggested that such phosphorylation might involve protein kinase C activated by phorbol ester. It is known that although protein kinase C and cAMP-dependent protein

kinase usually work in different secondary messenger signalling pathways they will act on the same protein and can cause similar cellular responses (131). Although the role of calpastatin phosphorylation has not been determined, it seems likely to bring about the redistribution of the inhibitor hence affecting the local distribution and thereby calpain activity.

Zimmerman and Schlaepfer (132) described the isolation of kinase activity associated with both calpain I and II from skeletal muscle. They observed that both calpain I and II were phosphorylated by this kinase in vitro and that the phosphorylation of calpain II was cAMP-dependent. Phosphorylation had no effect on the calcium sensitivity of the isoforms but reduced their specific activities. However Adachi et al (133) were unable to detect any phosphorylation of calpain I and II in vivo.

2.5.2. The Role of cAMP in the Control of Gene Expression at the Level of Transcription and Translation.

The modulation of enzyme activity by cAMP induced phosphorylation is not the only possible mechanisms by which altered activity could be seen. Cyclic AMP is also known to regulate gene expression and this could result in an increased activity of calpain and calpastatin. The work carried out in this thesis was undertaken in order to study the possible regulation of calpain and calpastatin activity at the level of transcription and translation.

Cyclic AMP-mediated regulation of gene expression has been implicated at various levels;

i) Transcription; various genes are thought to be regulated by 'cAMP-responsive regions' within promoters (134,135).

ii) Post-transcription/Pretranslation; this includes several steps involved in the processing of the primary transcripts into mRNA and its transport to the translational machinery. The stability of the mRNA is believed to be affected by changes in intracellular cAMP concentration (136-138).

iii) Translation; the production of the protein encoded by certain mRNAs is affected by changes in cAMP. Although this may not be a direct effect on the translational machinery, for example on phosphorylation of the ribosomal complexes, mRNA sequence or processing at levels i) and ii) can increase translationally efficiency (139).

Cyclic AMP-induced gene transcription.

Regulation of expression of a few genes has been shown to take place via activation of transcription through the cAMP responsive element (CRE). This is a sequence which is based on a palindromic motif, TGACGTCA (134). Various gene promoters have CREs within them and respond to cAMP, including somatostatin (140), proenkephalin (141), α human chorionic gonadotrophin (142) and β2-adrenergic receptor genes (143).

The exact characterisation of CREs has yet to be achieved; whether the CRE alone is sufficient to completely regulate gene transcription is not known. Several genes with 5' CREs have altered transcription rates when exposed to cAMP (134,135) including the β2-adrenergic receptor gene (143) which binds the protein involved in the activation of the CRE sequence, namely the CRE binding protein (CREB) (144). Other cAMP responsive element binding proteins have been identified, the binding protein CRE-BP1 (147) and a novel 120kDa binding protein (148). The CRE motif has been shown to be able to promote cAMP stimulation of transcription at a distance from the initiation codon in the DNA sequence and independent of orientation, a classification characteristic of an 'enhancer element'. Promoters are limited to the immediate 5' sequence of the gene, but enhancer sequences can also be located in this region. Some enhancers are required for basal transcription which is a characteristic of a promoter sequence, but they cannot induce gene transcription by themselves. There is evidence that CREs have dual role as both basal and inducible transcription enhancer elements (134,145,146). An example of this is the β2-adrenergic CRE sequence which when subjected to mutation removed transcription initiation activity from the promoter of the gene (143).

Both CRE-BP1 and CREB cDNAs indicate the presence of leucine zippers in their protein sequence (147,149). The leucine zipper is thought to be involved in the

dimerization of transcription activating proteins (150). For example the activator proteins Jun and Fos form a heterodimer, then bind to activator protein 1 (AP1) binding sites in promoters. Monomers of Fos will not bind to DNA but on forming a heterodimer with the protein Jun it will interact with DNA (150), suggesting a conformational change required for binding.

The CREB protein can be phosphorylated in vitro and in vivo. In vitro the phosphorylation is carried out by cAMP-dependent protein kinase (protein kinase A). The phosphorylated CREB will bind the CRE containing promoter of somatostatin and stimulate transcription as well as form dimers with transcriptional activity. Phosphorylation of CREB is essential for transcriptional activity, dimerization of the protein is also thought to be involved in the binding of CREB to DNA (151). Multiple phosphorylation sites for a variety of kinases have been identified in the CREB protein (149). However the implications of these plus the interaction of phosphorylation and dimerization have yet to be elucidated. Recent studies have shown that both CRE-BP1 and CREB will form heterodimers with the Jun protein (152,153) but whether this heterodimer formation takes place in vivo and any functional significance is not known. This heterodimer formation may play a role in transcription factor crosstalk allowing modulated expression of related genes.

The ability of cAMP induced phosphorylation to mediate changes in transcription allows rapid induction of mRNA for the genes without the need for de novo synthesis of the transcription activating factors. Most of the genes known to be activated by increases in cAMP do so very rapidly, the peak of transcript production is often less than a hour in cells in culture (134,135). The effects of long term exposure to cAMP analogues or stimulation of β-agonists on gene transcription has not been investigated in such experiments.

A different cAMP responsive sequence called the activator protein 2 (AP2) binding site has also been identified. The sequence is found in the promoters of several genes including metallothionein IIA (154), growth hormone (155) and Prolactin (156). Transcription from promoters containing AP2 elements is induced by phorbol esters and

forskolin, which activate protein kinase C and increase cAMP concentration respectively. The protein AP2 will bind AP2 elements in the promoter regions of genes containing the sequence (157).

Cyclic AMP-induced changes in mRNA stability.

Although certain genes have been shown to have increased transcription rates resulting from the activation of cAMP production, and the subsequent kinase-mediated activation of CREs or AP2 binding sites, there is evidence that cAMP also has an effect on mRNA stability.

Various mRNA have different half-lives some spanning several hours, like β-globulin, others, like c-fos, have a very short half-life of around 30 minutes in cell culture. The modification of the stability of mRNA would be a efficient way of increasing the production of a specific protein. The half-life of the mRNA transcripts for phosphoenolpyruvate carboxykinase (PEP-CK) is prolonged on the incubation of FTO-2B rat hepatoma cells with the cAMP analogue dibutyryl cAMP (Bt2-cAMP). There was no change in the stability of total cellular RNA indicating a specific effect on PEP-CK mRNA (137).

An example of increased degradation of mRNA, stimulated by the cAMP analogue 8-bromo-cyclic AMP (8-Br-cAMP), was seen in the increased turnover of mRNA for tyrosine aminotransferase in H-4 rat hepatoma cells (138). The mechanism of this cAMP-mediated regulation of mRNA stability is not understood.

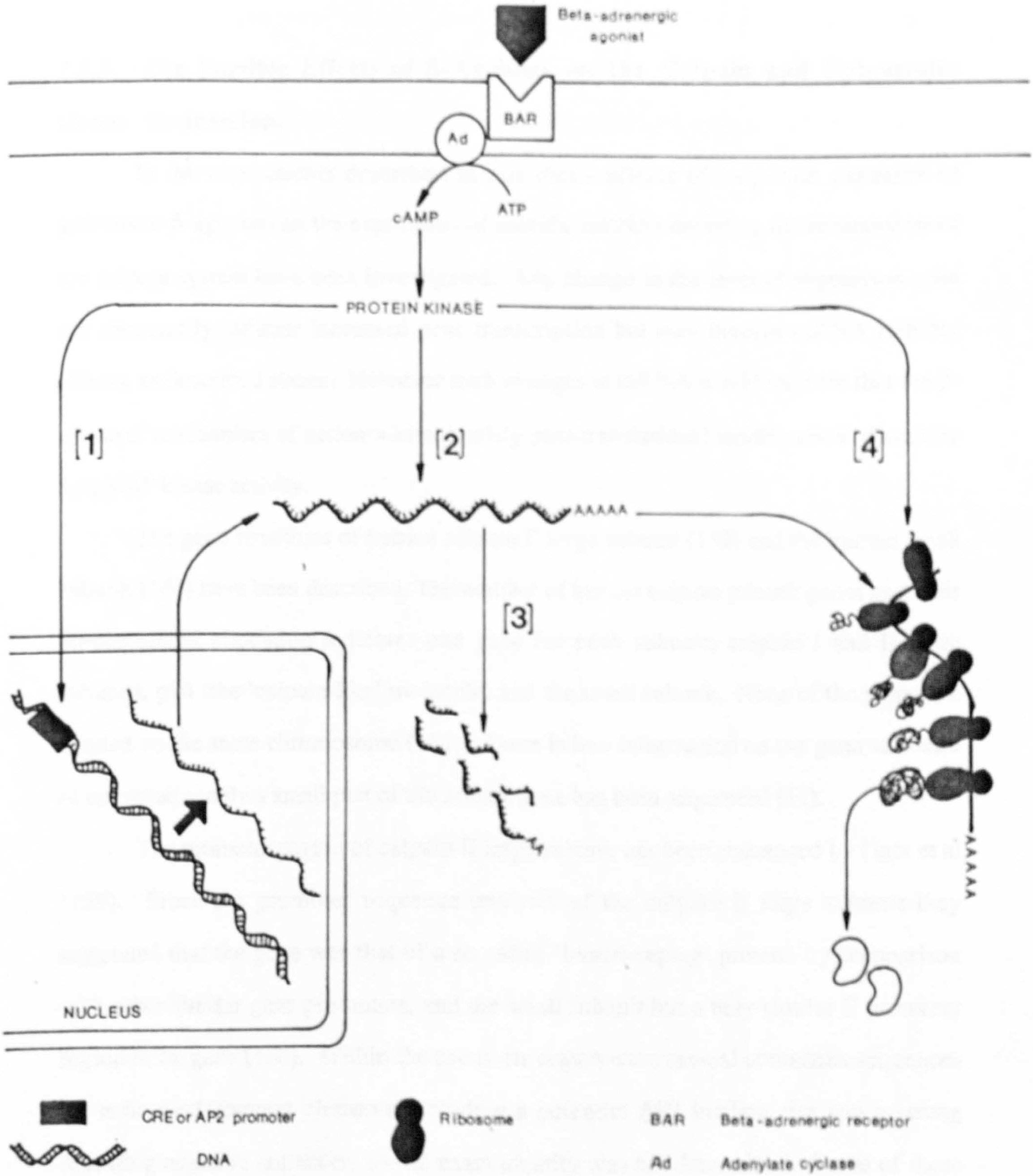
Lactate dehydrogenase expression is an example where the mechanisms of transcription, mRNA stability and efficiency of translation have been reported to be effected by cAMP (136). Stimulation of C6 glioma cells with either the β-agonist isoproterenol or dibutyryl cAMP lead to a two-fold increase in mRNA for lactate dehydrogenase cAMP, brought about by an increase in the half-life of the newly synthesised mRNA. Two populations of mRNA were identified, one with a half-life of 50 minutes the other at 2-5 hours (136). The suggestion that two different pools of lactate dehydrogenase mRNA were differentially regulated was also implied when the in vitro translation of the isolated mRNA was examined. There was a 8-10 fold increase in

lactate dehydrogenase mRNA expression caused by an increased efficiency of translation. Jungmann et al (136) suggested that the mRNA produced by cAMP induction was structurally different, although from Northern analysis of treated and nontreated cellular mRNA there was no difference in size. They believed the difference in the mRNAs was in their 3' noncoding regions and this gave the mRNA the potential for efficient translation. If this was the case the effect of cAMP was brought about by transcriptional or post-transcriptional modification of mRNA as the in vitro translation assay is independent of influence of cAMP on the translation machinery as might be seen in vivo. The studies on tyrosine aminotransferase or PEP-CK did not examine the translation efficiency of the mRNA they isolated.

The 3' untranslated region along with its poly(A)+ tail appears to be involved in the control of mRNA translation (158). Stability of mRNA is thought to be affected by the presence of the AU-rich motif which interacted with poly(A) binding proteins (PABP) normally thought to stabilise the mRNAs poly(A) tracts preventing their degradation. The consensus sequence UAUUUAU is found in the 3'untranslated region of mRNAs which target them to rapid turnover. Jungmann et al (136) suggested that the lactate dehydrogenase mRNA synthesised in the induced state was modified at the 3'nontranslated end which both affected stability and efficiency. The exact mechanism by which the untranslated regions target mRNA for varying rates of turnover as well as changes in translation efficiency is not known. The involvement of cAMP, although coincident with the changes in mRNA stability for the examples indicated, has not been positively shown to change the processes involved in mRNA turnover for cAMP induced changes in gene expression.

Figure 11 shows the various possible mechanism by which gene and resulting protein expression could be mediated by cAMP. Stimulation of β-adrenergic receptors by β-agonists causes the intracellular increase of cAMP. Increased transcription of specific genes is induced by elements associated with promoters that respond via CREs or activating protein 2 (AP2) binding sites [1]. The stability of mRNA is also affected by changes in intracellular cAMP [2], there being an increase or decrease in half-life of

Figure 11: A model for the regulation of gene expression by cAMP-mediated events.



mRNA mediated by changes in degradation [3]. It is not known if these changes are through protein kinase A activation. The efficiency of translation [4] may be due to a change in the mRNA characteristics mediated by cAMP causing transcriptional/post-transcriptional modifications resulting in altered translational efficiency.

2.5.3. The Possible Effects of β-Agonists on the Calpain and Calpastatin Genes Expression.

In the experiments described in this thesis effects of long-term exposure of animals to β-agonists on the expression of specific mRNAs encoding the components of the calpain system have been investigated. Any change in the level of expression need not necessarily indicate increased gene transcription but may involve mRNA stability effects, as described above. However such changes in mRNA would indicate that the β-agonists' mechanism of action was not solely post-translational modification of activity by cAMP kinase activity.

The gene structures of human calpain II large subunit (159) and the human small subunit (160) have been described. The number of human calpain subunit genes and their chromosomal allocation indicates one gene for each subunit; calpain I and II large subunits, p94 (the 'calpain-like' molecule) and the small subunit. None of the genes are located on the same chromosome (161). There is less information on the gene structure of calpastatin, only a small part of the human gene has been sequenced (61).

The promoter region of calpain II large subunit has been sequenced by Hata et al (159). From the promoter sequence analysis of the calpain II large subunit they suggested that the gene was that of a so called 'housekeeping' protein, by comparison with other similar gene promoters, and the small subunit has a very similar 5' promoter region in its gene (160). Within the promoter region were several consensus sequences for enhancer/promoter elements including a potential AP1 binding site and a strong repeating negative enhancer, whose exact identity was not determined. Some of these elements were also found in the small subunit gene. The AP1 binding site is under the influence of phorbol esters which stimulate protein kinase C. The response of calpain II

large subunit mRNA expression to phorbol esters has not yet been investigated, so whether the AP1 site does confer sensitivity to these agents is not known.

Hata et al (159) did not identify any CRE or AP2 consensus sequences, although they did not state whether they searched for such enhancer elements. From this work it appears that the gene may not be responsive to cAMP but, until the experiments carried out in this thesis, studies have not been carried out on the examination of calpain mRNA expression after stimulation through this secondary messenger system.

The promoter region of calpastatin gene has yet to be isolated, therefore its composition remains unknown. As with calpain mRNA there is no data concerning changes of the inhibitor mRNA expression in response to increases in intracellular cAMP.

Recent observations of Barka et al (162) have identified a cysteine proteinase inhibitor, cystatin S, whose mRNA and protein expression was induced in the salivary glands of rats when they were fed β-adrenergic agonists. The mRNA for this protein was undetectable in the glands of untreated rats but on treatment with β-agonists high levels of mRNA for the protein were detected. The rat salivary cystatin S gene has not been mapped but the gene in human salivary glands does contain a sequence similar to the CREs already isolated. Although cystatin S is a cysteine proteinase inhibitor there is no known relationship to calpastatin. The primary sequence of the pig inhibitor was compared against sequences of the 'cystatin superfamily' by Takano et al (53) especially the consensus inhibitory sequence gln-val-val-ala-gly which was identified in the cystatins (163). It was concluded that calpastatins are probably a different family of cysteine proteinase inhibitors which are specific to calpain (53). The cystatins are cysteine proteinase inhibitors but perhaps the only similarity to calpastatin is in their response to β-agonists. However one of the aims of the work carried out in this thesis was a similar investigation to that on cystatin S, namely whether β-agonists induced an increase in calpastatin mRNA.

2.5.4 Summary

Changes in cAMP could have effects on calpain and calpastatin activity. It appears that direct phosphorylation of calpastatin may have an effect on its subcellular distribution. The role of phosphorylation on the activity of the calpain system has not been comprehensively analysed particularly with respect to cAMP activated kinase effects on calpastatin in vitro and in vivo.

Cyclic AMP mediated effects on gene expression do appear to be a means by which increased expression of the components of the calpain system could be brought about, if the relevant genes do contain enhancer/promoter elements that respond to cAMP either directly or indirectly.

2.6. Overall Summary

The exact physiological role for calpain in skeletal muscle has yet to be determined. From in vitro studies the enzyme's substrates include some myofibrillar proteins and it is associated with states of skeletal muscle atrophy. However, calpain does not appear to be a general catabolic enzyme. Its proteolytic action may be to loosen myofibril structure initiating further protein degradation by other proteinases, or alternatively it could cleave other enzymes irreversibly 'activating' them with an ensuing catabolic effect.

β-agonist induced skeletal muscle hypertrophy appears to be achieved through decreased protein degradation and possibly an increase in protein synthesis. Although other proteinases may reduce their activity, the calpain system is the one which has been monitored and shown to have the capacity to decline in activity which may partially account for the diminished protein breakdown. This enzyme is also of interest because of its cytosolic distribution and its activation characteristics, being regulated by its own calcium sensitivity and a specific inhibitor. This apparently tightly regulated enzyme may be trigger for initiating further proteolysis.

The effects of β-agonists appear to be mediated through cAMP. As discussed above this could induce changes in activity of calpain and calpastatin at the level of direct phosphorylation or increased gene expression. The work in this thesis is concerned with the examination of the effects on gene expression at the mRNA level in β-agonist induced skeletal muscle hypertrophy, particularly the effects on the calpain system.

The following sections describe the techniques employed for the isolation of intact total RNA from skeletal muscle and the development of effective hybridization probes to be used to try and quantify any changes in the level of mRNA for calpain and calpastatin in the muscles of β-agonist treated animals.

Chapter 3. Materials and Methods

3.0 Materials.

The tissue samples used for the development of total RNA extraction techniques and the total RNA preparations used for DNA hybridization probe assessment were obtained from various sources within the Faculty.

Chicken tissue samples were from Ross broilers at 2-4 weeks old and fed a conventional starter diet. They were selected without reference to sex. Bovine (Friesian steers) and ovine (Suffolk x Clun Forest wethers) samples were obtained from animal trials within the Department. The steers were fed a 70% dry grass 30% barley pellet or a grass silage diet *ad libitum*, whilst the wethers were fed *ad libitum* on a pellet diet consisting of (g/kg): barley (225), oats (450), grassmeal (200), mineral mix (25) and Nutramol (Rumenco, Burton-on-Trent) (100). All the samples were removed from the animals directly after slaughter, frozen in liquid nitrogen then stored at -20°C or -40°C until used, which was up to a maximum period of 4 months.

All chemicals were analytical grade or electrophoresis grade, where available or relevant. For RNA and DNA manipulations chemicals and solutions were nucleic acid grade or they were at least analytical grade. The source of specialized chemicals, such as radioisotopes and enzymes are given in the text of the appropriate method. All other chemicals were obtained from BDH, Fisons or Sigma.

Solutions were made in sterile water and sterilized either by autoclaving at 120°C for 20 minutes or by passing through a 0.22µm sterile filter. Water used for nucleic acid manipulations was sterile, deionized and distilled. All glass and plastic laboratory equipment was soaked overnight in a 1M HCl acid bath before being cleaned thoroughly then rinsed in distilled water and was then sterilized by autoclaving.

Specialized equipment used in the course of the project included the following:

Pye SP8-400 UV/VIS Spectrophotometer.

Centrifuges; Beckman J2-21.

MSE 18

Bench top microfuge; Heraeus (Christ) Biofuge A.

Electrophoresis apparatus:

Polyacrylamide gels; Shandon Vertical Slab Unit.

Agarose gels; Bio-Rad, Mini-SubTM DNA Cell.

Gallenkamp Submarine gel systems, 'Maxi-cell' and 'Mini-cell'.

Sequencing gel; Bio-Rad, Sequi-Gen^{R} Nucleic Acid Sequencing Cell.

Power supplies;Bio-Rad power supply unit.

Chandos power pack.

Slot blot apparatus; Bio-Rad, Bio-Dot^{R} SF apparatus.

Dot blot apparatus; Bethesda Research Laboratories (BRL) Hybri-Dot Manifold.

PCR heating block; Hybaid.

Transilluminator; UVP Inc. Transilluminator.

Liquid scintillation counter; Intertechnique SL30 Liquid Scintillation Spectrophotometer.

All other apparatus was commonly used laboratory equipment such as water baths, pH meters etc.

3.1. Measurement of Calpain and Calpastatin Activity in Skeletal muscle.

The components of the calpain system, calpain I and II as well as calpastatin, were isolated from skeletal muscle by anion exchange chromatography on Fast Protein Liquid Chromatography (FPLC) Mono Q columns (Pharmacia) as in Higgins et al (114). Fresh muscle (2-4g) was finely chopped, or frozen tissue was ground to a powder in liquid nitrogen, then homogenized on ice with three volumes of buffer A (20mM Tris/HCl pH7.5, 5mM EDTA, 10mM 2-mercaptoethanol) on setting '5' of a Polytron homogenizer. After centrifugation for 20 minutes at 30000g and 4°C the supernatant of crude homogenate was filtered through 0.45µm then 0.22µm pore filters (Millipore). The filtrate was then loaded onto the Mono Q column which had been pre-equilibrated with buffer A. The column was washed with buffer A until the O.D. of the fractions eluting began to fall to the base line. Proteins were eluted with a salt gradient Buffer B (20mM Tris/HCl pH7.5, 5mM EDTA, 10mM 2-mercaptoethanol, 1.0M sodium chloride) was mixed with buffer A in a gradient from 0.1M upto 0.6M salt, over 20ml, using a FPLC programme.

Calpain and calpastatin activity was assessed in the fractions from the salt gradient based on the production of TCA-soluble peptides from casein originally described by Ishiura et al (235). The assay mix was 0.5ml consisting of; 0.2% (w/v) heat-denatured casein, 80mM sodium glycerophosphate pH7.5, 10mM 2-mercaptoethanol with 10mM calcium chloride or 10mM EDTA as a control to which was added 0.1ml of each fraction from across the salt gradient. The reaction was incubated at 30°C for 30 minutes, then the proteins precipitated by adding 0.5ml 10% (w/v) TCA. Calpain activity was detected as an increase in TCA soluble peptides in the presence of Ca^{2+} over the background in the absence of the ions as determined by absorbance at 280nm. The fractions containing activity were pooled and the total units of activity per kilogram of muscle determined after testing dilutions of the enzyme pool for linearity. One unit of enzyme activity was defined as the quantity of enzyme which produced an increase in absorbance at 280nm of one at 30°C for 30 minutes in this system.

Calpastatin activity was measured across the salt gradient using a similar assay system as that for calpain (114). Inhibition of pooled calpain II was determined in the presence of calcium ions. Each suspected inhibitor sample (0.1ml) was added to an assay tube containing a fixed quantity of active calpain II or heat-denatured inactive calpain (0.1ml) along with the assay solution (0.5ml) as described above. The decrease in absorbance at 280nm produced by the addition of a inhibitor sample was compared with a control of the change in absorbance produced by the calpain II alone, defined as 100% activity. Dilutions of the pooled inhibitor were used to obtain a linear regression line. From the relationship of the percentage inhibition against the quantity of inhibitor pool present the amount of inhibitor giving 50% inhibition of the calpain present was measured. One unit of inhibitor was the quantity required to inhibit one unit of calpain II activity at 30°C for 30 minutes.

These extraction procedures and assay conditions were modified for the analysis of the cimaterol bovine trial samples by introducing a hydrophobic chromatography step before FPLC as described by Etherington et al (236). To the supernatant of the centrifuged crude homogenate from 2g of muscle was added sodium chloride to a concentration of 0.3M. This was loaded onto a phenyl-sepharose (Pharmacia) column (2ml) pre-equilibrated with buffer A containing 0.3M sodium chloride. The calpastatin activity was washed through the column with an equal loading volume of buffer A, containing 0.3M sodium chloride. Calpain was then removed from the column using 50% ethylene glycol in buffer A (four column volumes, 8ml). The calpain and calpastatin pools were diluted fivefold with buffer A then each subjected to chromatograhy on the Mono Q FPLC column as described above.

From the salt gradient from the FPLC separation the location of the calpain I and II, along with calpastatin activity was determined and fractions pooled separately. The activity of the calpain isoforms and calpastatin were determined as described above. The inclusion of a hydrophobic step was reported to improve the separation of calpastatin and calpain which in some samples are incompletely resolved (236).

3.2. Extraction and Manipulation of RNA.

3.2.1. Extraction of Total RNA from Skeletal Muscle by a Phenol /Chloroform Based Method.

Skeletal muscle from the species chosen was dissected after slaughter, frozen in liquid nitrogen and then stored at -40°C or -20°C. Total RNA was extracted by a phenol/chloroform method which was modified for the preparation of total RNA from skeletal muscle (164). The muscle sample was ground to a powder in liquid nitrogen and up to 8g was used for extraction of total RNA. To the muscle powder was added 15 volumes of Homogenizing buffer; 50mM sodium chloride, 10mM magnesium acetate, 0.2M Tris-acetate pH8.5, 1.3% (v/v) Triton-X100 at 4°C. The mixture was homogenized on ice with a Polytron homogenizer at setting '5' for 15 seconds. To the homogenate was added SDS to 1% (w/v) and EDTA to 2mM, the mixture was then passed through two layers of muslin. The filtered homogenate was shaken for 10 minutes with an equal volume of phenol/chloroform 1:1 (v/v) - the phenol was saturated with Homogenizing buffer (30ml/100ml phenol) - for 10 minutes at room temperature. After centrifugation at 7,500g for 10 minutes at 20°C the upper aqueous phase was removed and stored on ice.

The interface and organic phase was then re-extracted with 15 times the original muscle weight of Extraction buffer; 0.1M sodium acetate, 2mM EDTA, 0.1M Tris-acetate pH9.0. The mixture was shaken for 5 minutes at room temperature then centrifuged as above. The aqueous phase was removed and combined with the first aqueous phase. Using a quarter volume (approximate 60ml) of phenol/chloroform 1:1, the combined aqueous phases were re-extracted by shaking at room temperature for 5 minutes. After re-centrifugation the aqueous phase containing the RNA was removed. Precipitation of RNA was carried out by adding 0.1 volume of 3M sodium acetate pH5.5 and 2.5 volumes of ethanol to the aqueous phase then leaving at -20°C overnight (165).

The RNA was recovered by centrifugation at 7,500g for 60 minutes at 4°C. The pellet was redissolved in water to approximately 1mg/ml and then cleaned to remove

contaminating DNA (165). An equal volume of 8M lithium chloride was added and the RNA left to precipitate for at least 2 hours at -20°C. RNA was recovered by centrifugation at 7,500g for 40 minutes at 4°C. The procedure was repeated once. Finally the RNA was reprecipitated twice with 0.1 volume of 3M potassium (or sodium) acetate pH 5.5 and 2.5 volumes of ethanol overnight at -20°C (section 3.2.4)

The RNA was recovered as described in the paragraph above and the pellet dried in a vacuum desiccator. The pellet was redissolved in water to 1mg/ml (2ml), re-extracted with phenol-chloroform and precipitated using 0.3M potassium acetate pH 5.5 and 2.5 volumes ethanol (section 3.2.4).

RNA pellets were redissolved to approximately 1mg/ml in water then stored at -70°C in suitable aliquots. The quantity of RNA was evaluated by spectrophotometric analysis at 260nm (section 3.2.5) and then examined on nondenaturing agarose electrophoresis (section 3.2.6).

3.2.2. Extraction of Total RNA from Skeletal Muscle by a Guanidinium Thiocyanate Based Method.

Total RNA was isolated according to the procedure of Chomczynski and Sacchi (166).

Muscle was ground to a powder under liquid nitrogen then homogenized for 15 seconds at room temperature in 10 volumes of solution D; 4M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% (w/v) sodium dodecyl sarcosinate, 0.1M 2-mercaptoethanol. It was then mixed with 0.1 volume of 2M sodium acetate pH 4, 1 volume phenol (water saturated) and 0.2 volume chloroform:iso-amyl alcohol mixture (49:1), with shaking after each addition . The acidic guanidinium thiocyanate-phenol-chloroform mixture was shaken for 10 seconds and then cooled on ice for 15 minutes. After centrifugation for 20 minutes at 4°C and 10,000g, the RNA was precipitated with 1 volume of isopropanol at -20°C for at least 1 hour. Centrifugation was repeated under the same conditions and the RNA pellet redissolved in 0.3 volume of solution D and reprecipitated with 1 volume of isopropanol at -20°C. Finally after centrifugation the

pellet was washed in 75% ethanol, re-centrifuged and then dried. The RNA was redissolved in water then quantified and examined as described in section 3.2.5 and 3.2.6.

3.2.3. Selection of Poly(A)+ RNA from Total RNA.

Poly(A)+ RNA was selected using affinity chromatography on a oligo-d(T) cellulose column (167,168). The Poly(A)+ RNA was isolated by a single or double passage through a column depending on the purity of Poly(A)+ RNA required. The appropriate quantity of oligo-d(T) cellulose (Pharmacia, Sigma), subject to its binding capacity and the amount of RNA to be loaded, was suspended in elution buffer; 10mM Tris-HCl pH 7.5, 1mM EDTA, and poured into a 1-5ml pasteur pipette column. The packed column was washed with 5 column volumes of binding buffer; 10mM Tris-HCl pH 7.5, 1mM EDTA, 0.5M sodium chloride, 0.1% (w/v) SDS. The total RNA was redissolved to 1-5mg/ml in water, heated to 65°C for 5 minutes, cooled, then diluted with an equal volume of double strength binding buffer.

RNA was applied to the column followed by one column volume of binding buffer once all the RNA had been loaded onto the column. The eluted fraction from the column was heated to 65°C for 5 minutes and reapplied; this was then repeated. When the RNA had been loaded the column was washed with 5-10 column volumes of binding buffer then 5 column volumes of wash buffer; 10mM Tris-HCl pH 7.5, 1mM EDTA, 0.1M sodium chloride.

Poly(A)+ RNA was eluted with 2-3 column volumes of elution buffer in less than 0.5 column volume fractions. To further enrich the Poly(A)+ RNA over Poly(A)- RNA the eluted fractions were heated to 65°C for three minutes and an equal volume of two times binding buffer added. The RNA was applied to the column, then washing and elution steps were repeated.

The Poly(A)+ RNA was precipitated from the eluted fractions as described in section 3.2.4. After being dried it was dissolved in water and stored at -70°C.

3.2.4. Extraction and Precipitation of RNA and DNA in solution.

Contaminants were removed from RNA and DNA in solution prior to use in various techniques, like PCR and in vitro translation, by phenol/chloroform extraction followed by ethanol precipitation (169).

An equal volume of phenol/chloroform (T.E. pH 7.6 saturated phenol, 3:10 T.E.:phenol - T.E. defined in appendix C) was added to the aqueous solution of RNA/DNA to be extracted. The solutions were vortexed for 10-15 seconds, then centrifuged for 3 minutes at 14,000g in a microfuge at room temperature. The upper aqueous phase was removed and the process repeated as required.

Nucleic acids in the aqueous phase were then precipitated.

For DNA 0.1 volume of 3M sodium acetate pH 5.5 was added followed by 2 volumes of ethanol. To precipitate the DNA the sample was either stored at -20°C overnight or at -70°C for 30 minutes. Centrifugation at 14,000 g for 15 minutes at 4°C pelleted the DNA and this was then washed in 70% ethanol by gently vortexing the pellet. After repeating the centrifugation for 10 minutes the pellet was dried in a vacuum desiccator then redissolved in water and stored at 4°C.

To prevent co-precipitation of dNTPs with DNA an alternative salt was used. Half a volume of 7.5M ammonium acetate was added to the sample followed by 2.5-3.0 volumes of ethanol, precipitation was carried out at -70°C for 15 minutes. This technique was not used directly before the use of phosphorylation or tailing enzymes as ammonium ions are reported to be inhibitory (169).

For RNA in aqueous solution which required precipitation ethanol was also used. One tenth volume of 3M sodium, potassium or ammonium acetate pH 5.5, or 0.1 volume of 8M lithium chloride along with 2.5 volumes of ethanol was added to the RNA sample. The solution was then treated as the DNA. The dried RNA was dissolved in water and stored at -20°C or long term at -70°C.

The choice of salt used was dependent on the application of the RNA in subsequent steps. For Poly(A)+ RNA purification, total RNA was precipitated with sodium acetate due to the presence of SDS in the oligo-d(T) cellulose chromatography

buffers. In vitro translations require the absence of sodium and chloride ions for efficient translation of mRNA, therefore the salt chosen was either potassium or ammonium acetate. Lithium ions were not used when the RNA was to be reverse transcribed as they inhibit the enzyme Reverse Transcriptase.

3.2.5. Determination of RNA and DNA Concentration in Solution.

The concentration, and to some degree the quality, of RNA and DNA dissolved in water could be assessed by scanning spectrophotometry in a 1cm path length quartz cuvette. Five to ten µl of RNA or DNA was diluted into 1ml of water and then scanned from 380 to 200nm. The concentration of the nucleic acid was determined by its absorbance at 260nm using the following values:

For DNA, an absorbance of 1 in a 1cm path length cuvette is approximately equivalent to 50µg/ml.

For RNA, an absorbance of 1 in a 1cm path length cuvette is approximately equivalent to 40µg/ml.

For oligonucleotides of known sequence the concentration for a 1cm path length was calculated using the sum of the extinction coefficients of each base (167):

dGTP = 11.7 ml/µmol

dCTP = 7.3 ml/µmol

dATP = 15.4 ml/µmol

dTTP = 8.8 ml/µmol

and the equation, Absorbance = ε.c.,

where ε is the extinction coefficient and c is the concentration.

3.2.6. Nondenaturing Agarose gel Electrophoresis.

Examination of RNA and DNA after extraction was carried out using agarose gel electrophoresis (170). The integrity of the rRNA bands in the RNA samples are to some extent an indication of the quality of the RNA. Electrophoresis was carried out using 1% (w/v) agarose for fragments greater than 1kb and 1.5% (w/v) agarose for fragments less

than 1.5kb, in 1xTBE buffer pH 8.3 (appendix C) in a mini gel system with a gel volume of 30 mls.

The nucleic acid was dissolved in water, 1-4µg in a volume of 10µl. Hind III or Eco RI/Hind III restriction enzyme digested lambda DNA markers (appendix B) were used to calibrate the gel. Approximately 0.5-1.0µg of marker DNA was used per track. To the samples was added 0.1 volume of loading buffer; 30% (w/v) Ficoll, 0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanole FF, 0.2M EDTA pH 8 in 10xTBE. The gel was loaded and run in 1xTBE for approximately 2 hours at 70 V.

To visualise the nucleic acid the gel was soaked in a 1µg/ml solution of ethidium bromide for 10 minutes, destained in water for 5 minutes, then placed on a transilluminator.

3.2.7. Denaturing Agarose Gel Electrophoresis:

i. Glyoxal Denaturing Gel System.

RNA was electrophoresed on 1% (w/v) agarose gel using the glyoxal denaturing method (171,172). A 16 well, 20x20cm, 250ml horizontal bed 1% (w/v) agarose gel was cast using 10mM sodium phosphate pH 6.5 as the gel buffer. RNA and DNA markers (less than 20µg RNA) were redissolved in 5µl of water and the following added;

		Final conc.
4µl	deionized glyoxal	1M
3µl	180mM sodium phosphate buffer pH 6.5	10mM
12µl	DMSO	50(v/v)

The mixture was incubated at 50°C for 1 hour, chilled on ice and 5µl of loading buffer; 0.05% (w/v) bromophenol blue, 0.05% (w/v) Xylene cyanole FF, 30% (w/v) Ficoll, 10mM sodium phosphate pH 6.5, was added. The gel was run at 100 V for 4 hours in 10mM sodium phosphate pH 6.5 until the leading dye was two thirds the way

down the gel. The running buffer was changed every hour to prevent deglyoxalation of the RNA due to the pH rising above pH 8.

Total RNA or DNA markers could be visualised after electrophoresis by staining the gel with 1µg/ml ethidium bromide then viewing with UV illumination. Glyoxal was removed from the gel by soaking for 30 minutes in 50mM sodium hydroxide, then for three 10 minute intervals in 50mM sodium phosphate pH 6.5. In the last wash ethidium bromide, 1µg/ml, was added to the buffer, the nucleic acid was then visualised by U.V on a transilluminator.

ii. Formaldehyde Denaturing Gel System.

A 16 well, 20x20cm, 250ml gel containing 1% (w/v) agarose, 6.6% (w/v) formaldehyde (2.2M) and 1xMOPS buffer pH 7 (appendix C) was cast (170,173,174). Appropriate amounts of RNA (less than 50µg) and approximately 2µg of Hind III digested lambda DNA markers were dissolved in 12µl of water and to these samples was added;

		Final conc
25µl	deionized Formamide	50% (v/v)
5µl	10xMOPS	1x
8µl	Formaldehyde (37% v/v)	6% (v/v)

The mixture was then incubated at 65°C for 5 minutes, chilled on ice and 5µl of loading buffer; 30% (w/v) Ficoll, 1mM EDTA, 0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene Cyanole FF, was added. The gel was run in 1xMOPS buffer overnight at approximately 30 V with one change of running buffer.

After electrophoresis the DNA markers were visualized by ethidium bromide staining (as described above), after first removing the formaldehyde from the gel by soaking it in 0.1M ammonium acetate, with several changes, for at least 2 hours.

For both glyoxal and formaldehyde gels DNA lambda markers (appendix B) and rRNAs, which were assumed to be approximately 4.7-4.8kb and 2.0-1.8kb for the 28S and 18S subunits respectively,were used as size indicators. The section of the gel

containing the size markers was cut away from the part to be used for Northern blotting so that the subsequent autoradiographs could be calibrated with the markers at a later data. The section was stained with ethidium bromide, after the denaturant had been removed, then viewed on a transilluminator as described in section 3.2.6..

3.2.8. Transfer of RNA to Nylon Membranes by Capillary Elution from Agarose Gels (Northern Blotting).

Northern blotting was carried out with nylon membranes (Hybond-N, Amersham) according to the protocol suggested by the manufacture (173).

The agarose gel which had been subjected to denaturing electrophoresis by one of the methods described in section 3.2.7, was cut to a minimal size. Two pieces of 3MM Whatman filter were cut to be a wick on which the gel was placed. The wick and the gel were supported above a reservoir buffer of 20xSSC or 20xSSPE (see appendix C). On top of the gel was placed the Hybond-N membrane which was the exact size of the gel. The buffer was drawn up through the gel by a stack of absorbent paper on top of the Hybond-N membrane. The stack consisted of 3 layers of 3MM Whatman filter paper above which were 2 layers of absorbent baby nappies. The stack was held in position by a 0.5-1kg weight, levelled to prevent uneven blotting. Care was taken not to short circuit the blotting procedure by the absorbent stack touching the wick or gel directly.

Blotting was carried out overnight, up to a period of 24 hours. Prolonging the blotting time does not significantly increase the quantity of RNA transferred (175). After blotting the membrane was removed from the gel and air dried. The RNA was then fixed to the membrane by UV transillumination for 4 minutes, then baked at 80°C for 2 hours. The membranes were stored at 4°C in sealed plastic bags.

3.2.9. Formamide-Formaldehyde Denaturing Dot/Slot Blotting.

RNA (less than 40µg) was dissolved in 40µl of water and mixed with;

		Final conc.
80µl	deionized formamide	50% (w/v)
28µl	formaldehyde (37% w/v)	7% (w/v)
8µl	20xSSC	1x

incubated at 68°C for 15 minutes, then cooled on ice. The samples were diluted using 1xSSC to give a series of half dilutions, the number of which varied according to the number of slots available. Once diluted 2 volumes of 20xSSC was added.

For dot blotting a piece of Hybond-N was cut to size and then placed on top of a piece of 3MM Whatman filter paper soaked in 10xSSC in the apparatus (BRL). Alternatively, using the slot blot apparatus (Bio-rad) a Hybond-N membrane was positioned as described by the manufacturer of the apparatus. For both dot and slot blotting a vacuum was applied so that the sample could be loaded onto the membrane then washed with 2x250µl of 10xSSC. The membrane was then removed from the apparatus and air-dried. The RNA was fixed to the Hybond-N and stored as previously described (section 3.2.8.).

3.2.10. <u>In Vitro</u> Translation.

<u>In vitro</u> translation was carried out using the rabbit reticulocyte lysate system (176,177). The lysate was produced by the John Innes Research Institute and was a gift from the Plant Physiology section within the Faculty. The stock lysate was endogenous mRNA-depleted and contained the following; potassium ions (as potassium chloride) at 35mM, magnesium ions (as magnesium chloride) at 2.4mM, calcium ions (as calcium chloride) at 3.0mM, calf liver tRNA at 50µg/ml (Bochringer Mannheim) and creatine phosphokinase at 6-12 U/ml (Sigma).

For the <u>in vitro</u> translation of mRNA the mixture contained the following:

		Final conc.
0.4µl	2M Potassium acetate (Amersham)	100mM
0.4µl	120mM Magnesium acetate (Amersham)	2.2mM
0.4µl	0.2M Creatine phosphate (Sigma)	8mM
0.4µl	1mM Amino acid mix minus L-methionine (Amersham)	0.04mM
0.5µl	0.2mg/ml Chloramphenicol (Sigma)	10µg/ml
1.0µl	10mCi/ml (>1000 Ci/mmol) L-(^{35}S)-Methionine <u>in vivo</u> labelling grade (Amersham)	< 10pmol
6.0µl	Rabbit Reticulocyte Lysate	60% (w/v)
1.0µl	Total RNA 1-4 µg/µl or water	0.1-0.4µg/ml

For each batch of translations all the components were mixed together in a pool of 'translation cocktail' then 9.1µl added to 1µl of total RNA or water, as a blank. The samples were incubated for 1.5 hours at 30°C. At the end of the time period 2µl of each translation was removed to determine the incorporation of ^{35}S into TCA precipitable protein. The volume was spotted onto a small square of Whatman No.1 filter paper and allowed to air dry. The filters were then submerged in the following:

i) Ice cold 10% (w/v) TCA, 1% (w/v) L-Methionine for 10 minutes,

ii) Boiling 10% (w/v) TCA for 10 minutes,

iii) 10% (w/v) TCA for 10 minutes,

iv) Ethanol for 2 minutes.

Filters were air dried, placed in 5ml of scintillation liquid and counted in the ^{14}C channel of a scintillation counter.

Using the values determined for ^{35}S incorporation equivalent quantities of counts (less than 100,000cpm/track) were loaded onto 1.5mm thick SDS-polyacrylamide gel (section 3.2.11.) from the remaining translation mix. Care was taken not to overload the gel. The maximum quantity of protein recommended for a 1.5mm gel is 1.5mg approximately equivalent to 15µl of lysate, as it contains up to 100mg/ml haemoglobin.

An equal volume of 'SDS-mix' (section 3.2.11.) was added to the lysate, the mixture was heated in a boiling water bath for 2 minutes and the samples then loaded along with non-radioactive molecular weight markers. The gel was run overnight at < 10mA until the indicator dye had reached the bottom of the gel.

Using 10% (v/v) glacial acetic acid, 10% (v/v) glycerol and 15% (v/v) propan-2-ol the gel was fixed then impregnated with autoradiography enhancer (En3hance^{TM}) for 1 hour, followed by washing in water for 30 minutes. Gel drying was carried out under vacuum at 65°C for 2 hours in a gel dryer (Bio-rad).

The gel was autoradiographed with Fuji RX film at -70°C.

3.2.11. SDS-Polyacrylamide Gel Electrophoresis.

Separation of radioactively labelled and non-labelled proteins was carried out by 8% or 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (178).

Gels were cast using a stock of 30% (w/v) acrylamide solution; 30% (w/v) acrylamide, 0.8% (w/v) N,N'-methylene bisacrylamide, in a 1.5mm thick, 20x20cm gel casting system using the following solutions;

	Volume for required % (w/v) acrylamide		Final conc.
	8%	10%	
30% (w/v) stock acrylamide	13.3ml	16.7ml	/
1M Tris-HCl pH8.8	19.0ml	19.0ml	0.38M
1% (w/v) SDS	5.0ml	5.0ml	0.1% (w/v)
TEMED	20µl	20µl	0.04% (v/v)

Water added up to a final volume of 50ml

To 10ml of the appropriate stock solution was added 20µl of extra TEMED followed by 0.5ml of freshly prepared 5% (w/v) ammonium persulphate. This was mixed and immediately used to produce a leak proof seal down the spacers and the

bottom of the gel. To the remaining gel solution was added 0.8ml 5% (w/v) ammonium persulphate, the gel then being cast so that enough space remained for the stacking gel, i.e. 2-3cm short of the top. Overlaying with water ensured a sharp interface on top of the gel.

After at least 2 hours the water on the top of the gel was removed and the stacking gel applied. This consisted of the following;

	Volume	Final conc.
30% (w/v) stock Acrylamide	1.7ml	5.1% (w/v)
1M Tris-HCl pH6.8	1.26ml	0.126M
1% (w/v) SDS	1.0ml	0.1% (w/v)
TEMED	20µl	0.2% (v/v)

Final volume made up to 10ml with water

Polymerization of the stacking gel was carried out with 0.4ml of 5% (w/v) ammonium persulphate. The solution was poured onto the gel and a 10mm or 5mm tooth comb inserted.

Once set the comb was removed and the gel placed in a electrophoresis apparatus with the running buffer; 190mM glycine, 25mM Tris, 0.1% (w/v) SDS. To the samples was added an equal volume of 'SDS-mix'; 0.125M Tris-HCl pH6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 1% (v/v) (1.4M) 2-mercaptoethanol. The mixture was heated for 2 minutes in a boiling water bath, then loaded onto the gel.

After running the gel at 30 mA through the day or 10 mA overnight it was fixed and stained (non-radioactive gels) in; 0.05% (w/v) coomassie blue, 0,05% (w/v) naphthol black, 10% (v/v) propan-2-ol, 15% (v/v) glacial acetic acid for at least 2 hours, then destained in 10% (v/v) glacial acetic acid.

3.2.12. Assessment of the Protein Concentration of Total RNA Samples.

The protein concentration of the samples containing extracted total RNA was measured using a protein assay kit (Bio-Rad) according to the manufacturer's instructions. The 'micro range' of protein determination was used. A standard curve was constructed using BSA in a suitable concentration range (0-25µg/µl).

3.3. Synthesis of Hybridization probes; Oligonucleotides and PCR Generated cDNA.

3.3.1. Synthesis of Oligonucleotides.

Oligonucleotides were made by the AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge or the Department of Applied Biochemistry and Food Science (Microbiology section), Faculty of Agricultural and Food Sciences, Nottingham University.

3.3.2. Labelling of Oligonucleotides by Polynucleotide kinase.

The oligonucleotides were dissolved in water to a concentration of 10pmol/µl and stored at -20°C. The oligonucleotides were supplied with a 5' hydroxyl group so that polynucleotide kinase was used to label directly without the need to dephosphorylate.

A typical reaction consisted of the following in 50µl;

		Final conc.
2µl	Oligonucleotide (20pmol)	0.4µM
10µl	100µCi [γ^{32}P]ATP (>5000 Ci/mmol) (<20pmol)	< 0.4µM
10µl	0.5M Tris-HCl pH7.6	0.1M
	0.1M Magnesium acetate	20mM
10µl	0.05M DTT	10mM
13µl	Water	/
5µl	Polynucleotide Kinase (4 U/µl)	20U

The reaction mix was incubated for 1 hour at 37°C.

The ratio of oligonucleotide to ATP was varied during the course of the development of labelling and hybridization experiments according to how much [γ-32P]ATP was available. The quantity of oligonucleotide used was 20-40pmol (0.25-0.5µg) of 39mer oligonucleotide I. This was labelled with either equimolar or a molar

ratio of 3:2 oligonucleotide to radioactively labelled ATP. Reducing the ATP concentration did not appear to affect its labelling as much as the age of the polynucleotide kinase. The enzyme seems to be unstable under the conditions used in the reaction.

The incorporation of ^{32}P into the oligonucleotide was monitored by denaturing polyacrylamide-urea electrophoresis of fractions eluted from a Sephadex G-50 column separation of the products (3.4.10. and 3.4.11.).

According to the number of filters to be probed, the volume of the oligonucleotide to be used as a probe was variable However the concentration of the probe in the hybridization solution was always 10-15ng/ml.

3.3.3. Labelling of Oligonucleotides by Specific Oligonucleotide Primer Extension.

An oligonucleotide complementary to the sequence required as a probe was used as a template and a smaller oligonucleotide complementary to the 3'end of the template as a primer. This was then extended 5' to 3' using the Klenow Fragment (Boehringer) (179).

The oligonucleotide was radioactively labelled using [α-^{32}P]dCTP (3000 Ci/mmol) (Amersham), with the following reaction components in a final volume of 20µl;

			Final conc.
2µl	Extension	600mM Tris-HCl pH 7.5	60mM
	buffer	600mM sodium chloride	60mM
		60mM Magnesium chloride	6mM
1µl		40mM DTT	2mM
1µl		Template oligonucleotide	/
		at approx. 50ng/µl	

Continued on following page

		Final conc.
1µl	Primer oligonucleotide at a 4 fold molar excess over template	/

This mixture was heated to 55°C for 10 minutes, cooled to room temperature, then the following added;

6µl	Trinucleotide mix each dNTP at 100µM	10µM (each dNTP)
5µl	50µCi [α-32P]dCTP (3000 Ci/mmol) 0.83µM	16.6pmol
1.5µl	Water	/

After equilibrating at 4°C for 5 minutes, the enzyme was added;

2.5µl	Klenow Fragment (2U/µl)	5 Units

The reaction was incubated on ice for 2-3 hours, stopped by the addition of 2µl of 0.2M EDTA pH8 and heated to 65°C for 5 minutes.

The incorporation of ^{32}P into the oligonucleotide was monitored by denaturing polyacrylamide-urea electrophoresis of fractions from Sephadex G-50 liquid chromatography separation of the products (3.4.10 and 3.4.11).

The quantity of the probe loaded per filter hybridization was < 5ng/ml.

3.3.4. Synthesis of cDNA by the Polymerase Chain Reaction (PCR)

Introduction.

First strand cDNA was synthesised from the mRNA of choice and a selected region was then specifically amplified by PCR (180-182). First strand cDNA complementary to mRNA was made using oligo-d(T) or a specific oligonucleotide as a primer and Reverse Transcriptase. The oligonucleotide primers were complementary to the 3'end of mRNA, so called 3'oligos. The complementary first strand cDNA was made 5' to 3' with respect to the DNA toward the 5' end of the mRNA.

The second PCR oligonucleotide primer (5'oligo), was located at the 5'end of the mRNA, complementary to the newly-synthesised first strand cDNA. The cDNA was amplified into double stranded cDNA between the 2 oligonucleotides using PCR. This is illustrated in Figure 24 (section 4.1.2.) where both means of priming first strand cDNA are shown along with the subsequent PCR amplification of the required sequence. Although oligo-d(T) was used as the primer for first strand cDNA it was not the 3'oligo of choice for PCR. A 3'oligo complementary to the mRNA sequence was used, 500 to 1000 bp downstream of the 5'oligo PCR primer.

The oligonucleotides used in PCR as primers were at least 16 nucleotides long, ideally 20-24 nucleotides. To aid cloning of the amplified sequence into vectors the PCR oligonucleotide usually had restriction endonuclease sequences added to their 5' termini plus some extra 'nonsense' nucleotides to facilitate restriction digest (167).

PCR amplification of cDNA made from mRNA was carried out by one of two methods as outlined below.

i. Method I: PCR amplification of AMV reverse transcriptase generated first strand cDNA.

Synthesis of first strand cDNA from mRNA was achieved using AMV Reverse Transcriptase (NBL), oligo-d(T) (or a specific 3'PCR oligonucleotide) and either 4µg poly(A)+ RNA or 10µg total RNA (183).

For the reaction AMV was diluted 10 fold in;

10% glycerol

10mM potassium phosphate pH 7.4

0.2% Triton X-100

2mM DTT

and then equilibrated on ice for 30 minutes.

The reaction was carried out in a final volume of 40µl consisting of the following:

		Final conc
	Tris-HCl pH 8.3	50mM
AMV	Potassium chloride	50mM
buffer	Magnesium chloride	10mM
	EDTA	1mM
	DTT	1mM
	BSA, Nucleic Acid grade (Pharmacia)	10µg/ml
	Oligo-d(T)12-18 (Pharmacia)	
	or specific 3'PCR oligo	10µg/ml
	dNTPs (Pharmacia)	each at 1mM
	Spermidine-HCl	0.5mM
	Sodium pyrophosphate	4mM
	RNAasin (Pharmacia)	40-80 Units
	Dilute AMV Reverse	
	Transcriptase	10 Units
	Poly(A)+ RNA	0.1µg/µl
	or total RNA	0.25µg/µl

The RNA was diluted into 10µl and heated to 70°C for 3-5 minutes before it was added to the reaction mix. Two to four µl of the reaction mix was removed and 'spiked' with 1-2µCi [α-32P]dCTP, to monitor the progress of the reaction at the end of the incubation period on a denaturing polyacrylamide/urea gel (section 3.4.11.).

The reaction mixture was incubated at 42°C for 45 minutes, then cleaned once with an equal volume of phenol (TE saturated pH7.6) and then with phenol/chloroform and precipitated with ethanol and 2.5M ammonium acetate (section 3.2.4).

Using 20 and 200ng of the first strand cDNA, generated by a specific 3' PCR oligo or oligo-d(T) primers, the cDNA sequence was amplified via PCR with 5' and 3' PCR oligonucleotides 500-1000 bp apart (184), as described in section 4.1.2..

PCR was carried out with the following components in a final volume of 100µl;

		Final conc.
PCR buffer	Potassium chloride	50mM
	Tris-HCl pH 8.4	10mM
	Magnesium chloride	2.5mM
	Gelatin	200µg/ml
	dNTPs (Pharmacia)	each at 0.2mM
	5' oligonucleotide	100pmol
	3' oligonucleotide	100pmol
	First strand cDNA	20ng or 200ng

This was overlaid with 100µl of mineral oil (Sigma). The reaction was incubated in a programmable heating block (Hybaid). The mix was heated to 95°C for 5 minutes to denature the nucleic acid, cooled to 50°C then incubated for 2 minutes to allow annealing of the oligonucleotides. At this point 2 units of Taq polymerase was added. The heating block then raised the temperature to 68-70°C for 15 minutes to allow elongation of the primers to take place. Subsequent cycles consisted of; 1 min at 95°C, 2 min at 50°C and 15 min at 68-70°C. The cycle was repeated for 35-40 times. At the end of the cycling period the temperature was held at 70°C for 30 min to allow reannealing of the products.

The mineral oil was removed and 5-10µl of the PCR solution analysed on an agarose nondenaturing gel (3.2.6.). The remaining mixture was phenol/chloroform extracted and ethanol precipitated (section 3.2.4.).

II. Method II: Direct PCR amplification of MMLV reverse transcriptase generated first strand cDNA without intermediate precipitation.

I acknowledge the help of Dr R.S.Gilmour in the application of this technique developed at the laboratories of the AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge.

Total RNA (20µg) was first cleaned by extraction twice with phenol/chloroform and precipitated with ethanol plus 0.3M potassium acetate (section 3.2.4). The dried pellet was redissolved in 10µl of water with 15-30ng of oligo d(T)12-18. Annealing of the oligo-d(T) to the poly(A) tracts of the mRNA was carried out by heating the RNA to 75°C for 5 minutes, to denature secondary structure, then cooling to 37°C.

To the oligo-d(T) annealed RNA were added the components of the reverse transcriptase reaction in a final volume of 20µl;

		Final conc
MMLV buffer	Tris-HCl pH 8.3	50mM
	Magnesium chloride	8mM
	DTT	10mM
BSA, Nucleic acid grade (Pharmacia)		0.25µg/µl
dNTPs (Pharmacia)		each at 2.5mM
RNAasin (Pharmacia)		20 UNITS
MMLV reverse transcriptase (Pharmacia)		40 UNITS

The reaction was incubated at 37°C for 2 hours. At the end of the incubation the 20µl was diluted to 100µl with water. From this dilute volume was taken 0.2, 0.4 and 10µl to be used in PCR. These volumes were further diluted into the 100µl of PCR reaction mix consisting of the same components as described in section 3.3.4.(i). PCR temperature cycling was carried out as described above (section 3.3.4.(i)) with 2 units of Taq polymerase, for 35-40 cycles. The products were examined on nondenaturing

agarose gel (section 3.2.6). The remaining solution was then extracted and precipitated as previously described (section 3.2.4).

3.4 cDNA Manipulation, Probe Labelling, Hybridization.

3.4.1. Ligation of Cohesive Termini.

Plasmid and the DNA to be ligated into it were digested with restriction endonucleases, the fragments with the cohesive termini being isolated by nondenaturing agarose gel electrophoresis and electroelution (section 3.2.6 and 3.4.7.). Approximately 50ng of plasmid was used in ligation reactions with the DNA to be ligated into the vector at 1:1 and/or 3:1, DNA:vector molar ratio (167,187).

The reaction was carried out in a final volume of 10µl with the appropriate molar ratio of cDNA to plasmid in the following: Ligation buffer (20mM Tris·HCl pH 7.6, 10mM Magnesium chloride, 10mM DTT), 0.6mM ATP, 5ng/µl plasmid and 1 unit T4 DNA Ligase (Boehringer). It was incubated for 1 hour at 22°C, then chilled on ice before being used in the transformation of competent cells (see below).

Where cohesive ends of a plasmid were to be religated, 100ng of plasmid was used in the reaction volume above, with no foreign DNA present.

3.4.2. Transformation of E.coli.

Competent Cells were produced using a modified calcium chloride procedure (169). A 4ml LB culture (appendix D) of the appropriate strain of E.coli was grown overnight at 37°C from a colony off stock minimal plates. Using 0.5ml of the overnight culture 50ml of LB broth was inoculated, incubated to an absorbance of 0.55 at 600nm, then cooled on ice. From the chilled media 20ml was taken and centrifuged for 7 minutes at 3,750g at 4°C. All the supernatant was carefully removed then, on ice, 2ml of ice-cold 0.1M calcium chloride was added. The cells were gently resuspended and left to incubate, on ice, for 1 hour. After incubation 0.2ml of the cells was removed and 5µl of the ligation mix from the ligation reaction (25-50ng DNA) was added. The cells were then left for 30 minutes on ice, after which they were 'heat-shocked' at 42°C for 3 minutes, then placed back on ice for 20 minutes. To this suspension of transformed cells was added 0.8ml of LB broth and the cells were then incubated with shaking at 37°C for

45 minutes. The cells were plated out onto LB plates containing the appropriate antibiotic using a range of volumes of the culture; 50, 100 and 200µl.

3.4.3. Growth of E. coli for the Preparation of Plasmids

Cells containing the required plasmid, for example those from frozen glycerol stocks (see below) were plated out onto LB agar plates (appendix D) containing the appropriate antibiotic; 50µg/ml Ampicillin or 12.5µg/ml Tetracycline (169,186). Alternatively for freshly transformed cells a variety of culture volumes were used; 50, 100 and 200µl.

Cells were allowed to grow overnight or until colonies became distinct. Individual colonies were then 'picked-off', 4ml of LB broth containing the relevant antibiotic inoculated and the medium incubated overnight in a rotary shaker at 37°C. The cells were then used for mini-preps (section 3.4.4) or for inoculation and subsequent overnight incubation of up to 1 litre of LB broth containing antibiotic for large scale plasmid preparations.

Small LB cultures (4ml) were also used for storage cultures; glycerol was added to 15-20%, the medium was mixed and then stored at -70°C in 1ml aliquots.

3.4.4. Small Scale Plasmid Preparations; 'Mini-Preps'.

Mini-preps were carried out using the method of Maniatis et al (167). This included the phenol/chloroform extraction of the DNA in the supernatant of the bacterial lysate (centrifuged to remove cell debris) before ethanol precipitation of the DNA. This allowed easier restriction endonuclease digest analysis.

3.4.5. Large Scale Preparations of Plasmids; 'Maxi-Preps'

Maxi-preps were carried out using a Qiagen plasmid kit with Qiagen 500 columns (Hybaid) according to the manufacturer's specifications.

3.4.6. Restriction Endonuclease Digest of DNA

Using the appropriate restriction endonuclease (NBL, Bochringer) and their buffers (as supplied), various quantities of DNA were digested. For the preparation of cDNA inserts from plasmids, 50-100µg of DNA was subjected to restriction endonuclease digestion at approximately 1 Unit/µg of DNA in 100µl of solution with the appropriate buffer.

For less than 10µg of DNA, reaction volumes were 20µl with the minimum of approximately 10 Units of enzyme in the relevant buffer. With multiple restriction endonuclease digests the reactions were carried out in succession with ethanol precipitation between each step.

Most restriction endonuclease reactions were incubated at 37°C for 2 hours. Care was taken to minimize 'starring activity' of certain restriction endonucleases, such as Eco RI, by keeping the digest times as short as possible and the enzyme concentration low (187).

3.4.7. Preparation of cDNA Inserts from Plasmids

After digestion of the plasmid with the relevant restriction endonuclease to excise the cDNA insert, the DNA was subjected to nondenaturing agarose gel electrophoresis, stained with ethidium bromide and the cDNA insert cut from the gel. The cDNA insert in the gel was placed into dialysis tubing, prepared by boiling in 2% (w/v) sodium hydrogen carbonate and 1mM EDTA for 10 minutes, then soaking in water for 10 minutes. The dialysis tubing was filled with 0.5xTBE and sealed. DNA was then electroeluted from the gel in 0.5xTBE running buffer at 100 V for 4 hours. Before switching off the current the polarity was reversed for 5 minutes. The DNA solution was then removed from the dialysis bag, phenol/chloroform extracted and ethanol precipitated (section 3.2.4). The dried DNA was redissolved in water and stored at 4°C.

3.4.8. DNA Sequencing

DNA sequencing was carried out using a T7Sequencing kit (Pharmacia). The kit uses the dideoxy method of sequencing (188). The procedure was carried out as specified by the manufacturer using ^{35}S in the form of [α-^{35}S]dATPαS to label the products. The reaction procedure is summarized below.

For each reaction 1.5-2µg of plasmid containing the cDNA to be sequenced was used. This was alkali denatured with sodium hydroxide at a final concentration of 0.4M. After incubation at room temperature for 10 minutes the DNA was precipitated with ethanol on ice, centrifuged to a pellet, washed in 70% ethanol, then briefly dried. The pellet was redissolved in 10µl of water and annealed to the appropriate quantity of primer. In most cases this was 10:1 primer to template ratio. Annealing was carried out using the solution and instructions provided in the kit, as were the labelling and termination reactions. Once annealed the primer was extended and labelled in a 5' to 3' direction using T7 polymerase, a mixture of dNTPs and [$\alpha^{35}S$]dATPαS (> 1000 Ci/mmol) (Amersham) at room temperature for 5 minutes. The labelling mix was then split into 4, an equal volume going to one of four terminating solutions which contained the deoxynucleotides (dNTP) for extension, but each having a different dideoxynucleotide (ddNTP) present. The four reactions were incubated at 37°C for 5 minutes, the elongating fragments being terminated by the appropriate ddNTP at different lengths where the corresponding dNTP would have occurred in the sequence. Either 'long' or 'short' ddNTP/dNTPs terminating reaction mixes were used. These produced up to 1000 or 500 bp fragments respectively by varying the ratio of ddNTP to its corresponding dNTP present in each solution.

At the end of the incubation period the reaction was terminated by adding the 'STOP' loading buffer provided in the kit. The products of the four reactions were then subjected to electrophoresis on sequencing gels.

Denaturing polyacrylamide-urea electrophoresis was carried out on a Sequi-Gen Nucleic Acid sequencing cell system (Bio-Rad) 40x21cm, using different strengths of polyacrylamide (5-8%) 0.4mm thick gels according to the manufacturer's instructions.

The electrophoresis apparatus glass plates were thoroughly cleaned by washing with 0.1% (w/v) SDS, ethanol, 0.5M sodium hydroxide and water in succession, then dried. The non-notched plate was then coated with binding silane; 0.1% (v/v) delta-methacryloxypropyl trimethoxysilane (Sigma), 1.75% (v/v) glacial acetic acid in 5ml ethanol, allowed to dry, wiped with ethanol and then polished. The notched plate was coated with 10 ml ethanol containing 5% (v/v) Sigmacote (Sigma), allowed to dry then polished thoroughly. The apparatus was assembled with 0.4mm spacers.

A 60ml solution containing the appropriate % acrylamide- 7M urea was made according to the following;

	Quantities required for the strength % (w/v) acrylamide gel		
	5%	6%	8%
40% (w/v) stock acrylamide	7.5ml	9ml	12ml
10xTBE	6ml	6ml	6ml
Urea	25.2g	25.2g	25.2g

To seal the bottom of the gel a filter wick was soaked with 10ml of acrylamide/urea solution and polymerised with 125µl 10% (w/v) ammonium persulphate, 50µl TEMED. The bottom of the gel was placed on top of the wick, the polymerizing solution being drawn into the gap to seal it.

To cast the gel the remaining solution was poured from the top, polymerized with 150µl 10% (w/v) ammonium persulphate and 60µl TEMED. A sharks tooth or square-well comb was inserted into the gel and it was allowed to set for at least 2 hours at a 5^{o} inclination.

Before loading the sequencing reaction samples the wells were washed out and the gel was prerun at 40 W in a running buffer of 1xTBE for 30 minutes. Three microlitres of each of the four sequencing reactions was removed from the relevant stock solution and heated to 75-80°C for 2 minutes then 2µl immediately loaded onto the gel,

side by side, in the terminating ddNTP order of T, C, G, A. The gel was run at 40 W until the Xylene cyanole FF dye had run to the bottom, then the second loading was applied onto the gel. The last loading, either the second or third, was allowed to migrate until the bromophenol blue was at or near the bottom of the gel. The current was switched off, the plates carefully separated and the plate with the gel attached was soaked for 20-30 minutes in 10% (v/v) glacial acetic acid, 10% (v/v) methanol in distilled water. The gel was air-dried overnight or at 55°C for 3 hours. Once dry it was autoradiographed using HyperfilmTM-βmax (Amersham) for 1-2 nights at room temperature.

3.4.9. Preparation of Radioactively Labelled cDNA Probes:

Two methods of cDNA probe labelling were used;

1. Nick Translation.

This was carried out using a Nick Translation Kit (Amersham) and [α-^{32}P]dCTP 3000 Ci/mmol (Amersham). According to the manufacturers of the kit the maximal specific activity achieved is 0.5×10^8 dpm/µg using 1-2µg DNA and 50µCi of [α-^{32}P] dCTP (3000Ci/mmol).

In the reaction 1-2µg of DNA was used in 100µl of the following reaction mix;

		Final conc.
10µl	1-2µg DNA	0.01-0.02µg/µl
20µl	Trinucleotide mix (each dNTP at 100µM)	20µM (each dNTP)
5µl	50µCi [α-^{32}P]dCTP (3000Ci/mmol)	0.167µM
55µl	Water	/
10µl	Enzyme solution Pol I	5 Units
	DNAase I	100 pg

The reaction was incubated at 37°C for 1 hour, diluted to 100µl with TE buffer pH 7.6 and the reaction products separated by chromatography on Sephadex G-50 (section 3.4.10).

3.4.10. Chromatography of Radioactively-labelled Nucleic Acid on Sephadex G-50 and Assessment of 32P Incorporation.

The products of radioactive labelling reactions, ^{32}P labelled cDNAs and oligonucleotides, were separated from unincorporated isotope by chromatography through Sephadex G-50. Small columns, 5-6cm in length, were made in pasteur pipettes with Sephadex G-50 suspended in TE pH 7.6. The radioactive labelling reaction products were loaded onto the column once it had been washed in 4 column volumes of TE buffer pH 7.6. The column was then washed with TE buffer pH 7.6 and fractions of approximately 200µl were collected until all the activity had passed through judged by a geiger counter. From each fraction 2µl was taken and placed in 5ml of scintillation fluid then counted in a scintillation counter in a preset ^{32}P channel. From a graph of counts present (cpm) against the fraction number an indication of the efficiency of the isotope incorporation could be made by comparison of the relative areas of incorporated and unincorporated isotope. Figure 12 shows a typical separation. A clear peak of labelled probe can be seen as the first peak.

Those fractions which showed incorporation of ^{32}P into probe were pooled and 2µl of this counted to determine the approximate specific activity of the probe (cpm/µg). Using the total activity of the pooled probe the volume of the pool to be used for Northern or dot/slot blot hybridization was evaluated. The activity required for probe hybridization was approximately $1\text{-}4\times10^{7}$cpm/50ml, the exact amount varying according to the probe used. The concentration of the probe in the hybridization solution depended on the labelling procedure used as the specific activity of nick translated probes was less than that of random prime labelled probes, approximately 10 fold less. For random prime probes the concentration in hybridization solutions was approximately 1ng/ml, for nick translation probes approximately 10-15ng/ml.

The reaction was incubated at 10-15°C for over one and half hours. At the end of the incubation period the reaction products were separated by chromatography on a Sephadex G-50 column (section 3.4.10).

II. Random Prime Labelling.

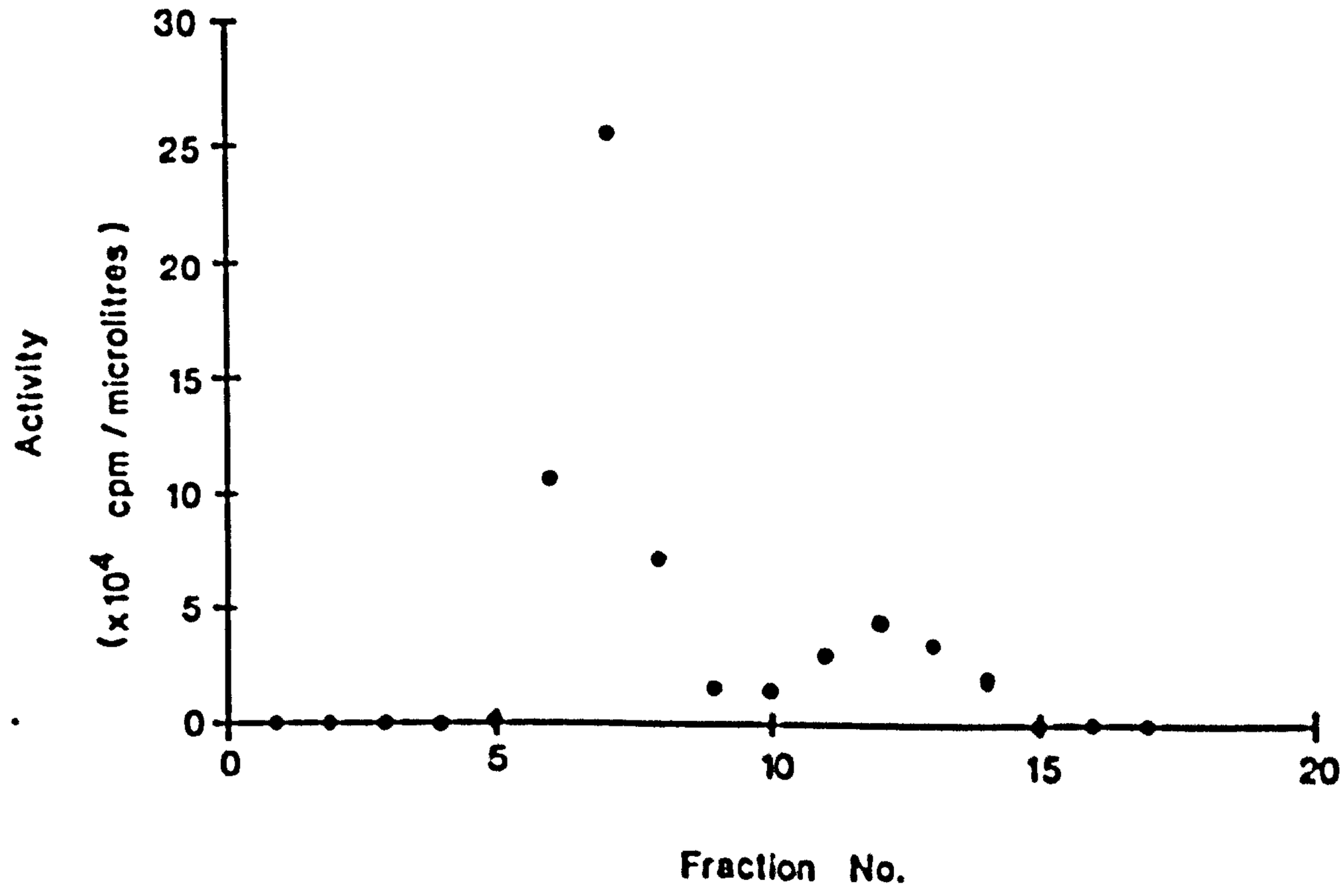
This method uses less cDNA and is able to produce probes of higher specific activity than nick translation. Unlike nick translation, in random primed reactions a new complementary strand is synthesised from a range of hexanucleotide primers by Klenow Fragment According to the manufacturers of the random primed DNA labelling Kit (Boehringer) it should give 1.8×10^9 dpm/µg when using 50µCi of [α-^{32}P]dCTP (3000 Ci/mmol) (Amersham) and 25ng of DNA. However the more DNA used the lower the specific activity achieved. Accordingly with 2µg the activity is 0.3×10^8 dpm/µg, near that of nick translation.

When using this method the quantity of probe to be labelled and the amount of activity used for hybridization techniques was carefully assessed to prevent high background on filters. For labelling experiments 25-50ng (or less) of the cDNA was used per reaction. This was approximately assessed as the quantity of cDNA insert just visible on a agarose nondenaturing gel stained with ethidium bromide.

DNA was first heat denatured at 95°C for 10 minutes, then the reaction was carried out in a final volume of 20µl with the following components;

		Final conc.
9µl	25-50ng cDNA	1.25ng/µl
3µl	Trinucleotide mix each dNTP at 0.167mM	25µM (each dNTP)
2µl	10x Reaction Buffer plus random hexanucleotide mix	1x
5µl	50µCi [α-^{32}P]dCTP 3000 Ci/mmol	0.83µM
1µl	Klenow Fragment	2 Unit

Figure 12: The Sephadex G-50 separation of ^{32}P random prime labelled human calpain II large subunit cDNA (0.1µg). The cDNA was labelled using a random primer labelling kit using the conditions as described in section 3.4.9.II.. The radioactively labelled products were separated on a Sephadex G-50 column and 2µl of each 200 µl fraction was assessed for activity (section 3.4.10.). Fractions 6-8 were used for hybridization experiments.



3.4.11. Denaturing Polyacrylamide-Urea Denaturing Gel Electrophoresis (UREA-PAGE).

To check incorporation of radioisotpe into oligonucleotides and some cDNA probes, samples were subjected to denaturing gel electrophoresis (170), after separation on Sephadex G-50 (section 3.4.10).

For 30-40 mer oligonucleotides 20% (w/v) acrylamide-7M urea gels were used, for longer sequences the percentage of acrylamide was reduced down to 3.5%.

Gels were cast using a stock of 40% acrylamide solution; 38% (w/v) acrylamide, 2% (w/v) N,N'-methylene bisacrylamide, according to the following;

	Quantity for final gel of required % (w/v) acrylamide		Final conc.
	20%	3.5%	
40% (w/v) stock acrylamide	25ml	4.38ml	/
Urea	21g	21g	7M
10xTBE	5ml	5ml	1x

Water was added to a final of volume of 50ml

To the appropriate solution was added 50µl TEMED. A plug to seal the gel was made using 10ml of the 50ml stock, to which was added an extra 10µl TEMED and then polymerized with 0.4ml of 10% (w/v) ammonium persulphate. Once the sealing strip had set the main gel was cast by adding 1.3ml of 10% (w/v) ammonium persulphate to the remaining 40ml of stock solution. A suitable size comb was inserted directly into the gel. Polymerization was allowed to take place for at least 2 hours.

The gel was run in a reservoir buffer of 1xTBE. Two to five µl samples of fractions were diluted with 15µl formamide and 0.1 volume of marker dye solution (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanole FF) was added. The loading mix was heated to 100°C for 2 minutes, then loaded on to the gel. After running at 20 W, until the bromophenol blue had migrated two-thirds the way down, the wet gel

was removed from the apparatus, covered with cling film and autoradiographed directly with Kodak AR or Fuji RX film for a time period according to the quantity of isotope loaded.

3.4.12. Northern and Dot/Slot Blot Filter Hybridization with Radioactively Labelled Probes:

i. Prehybridization.

Nylon Hybond-N membranes to be hybridized with radioactive labelled probes were first prehybridized according to the manufacturer's recommendations (Amersham).

The prehybridization solution consisted of: 6xSSC, 5xDenhardt's solution - 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) Polyvinylpyrollidone and 0.5% (w/v) SDS. To this was added sonicated salmon sperm DNA (Sigma) heat denatured at 100°C for 10 minutes to a concentration 0.1mg/ml. For Northern blots of up to 400cm^2, 40-50mls of prehybridization solution was used; for slot/dot blots up to 120cm^2, 20-25mls.

An alternative system used for prehybridization replaced 6xSSC with 5xSSPE and included 50% formamide in the solution shown above. The same volumes for prehybridization were used as above.

For both solutions the appropriate volume was added to the membrane in a plastic bag which was sealed so that it contained the minimal number of air bubbles. For solutions containing SSC, prehybridization was carried out at 55°C. For those with formamide present 42°C was used. Both were left overnight (up to 18 hours).

ii Hybridization.

After prehybridization the predetermined quantity of radioactively-labelled probe was added to the prehybridization solution, following its separation by chromatography and an assessment of isotope incorporation, as described in section 3.4.10.

The double stranded cDNA probes were denatured by heating to 100°C for 5-10 minutes. The probe was then added to and thoroughly mixed with the prehybridization solution which became the hybridization solution. Care was taken not to introduce the probe directly onto the membrane and air bubbles into the bag. Hybridization was

allowed to take place at 55°C overnight (up to 24 hours). For hybridization solutions containing formamide, the incubation temperature was 42°C.

iii Posthybridization Washing and Autoradiography.

After hybridization the membrane was washed at a set temperature and salt concentration. The first wash was at the hybridization temperature and salt concentration, 55°C and 6xSSC for 20 minutes, then repeated. The membrane was autoradiographed at -70°C with Kodak AR or Fuji RX film with two intensifying screens in an autoradiography cassette. Care was taken not to allow the membrane to dry out when washing or during autoradiography.

After the initial wash the membrane was treated at a higher stringency by increasing temperature in 5°C increments and lowering the salt concentraton and where appropriate incorporating SDS into the washing procedure. The washes were carried out for two periods of 15-20 minutes. The conditions of subsequent washing at increasingly higher stringency were dependent on the activity on the membrane. The highest stringency wash was at 65°C with 0.1xSSC, 0.1% (w/v) SDS.

For hybridizations done in 50% formamide the membranes were washed in a similar fashion. The first wash was at room temperature with 5xSSPE, 0.1% (w/v) SDS, then the temperature was increased to 42°C for subsequent washes with decreasing salt concentration.

3.4.13. Removal of Hybridization Probes from Northern Blots.

Initial methods involved soaking the membrane in boiling 0.1% (w/v) SDS, allowing the solution to cool, then repeating this once. The Northern was then autoradiographed to check for the removal of the radioactive probe. The procedure was repeated if activity was still visible on the membrane filter.

The boiling solutions used in the procedure described above had a tendency to produce membrane damage. An alternative method was therefore adopted. The Northern was immersed in 50% formamide, 2xSSPE for at least 1 hour at 65°C. After incubation the membrane was rinsed in 0.1xSSPE twice. The damp membrane was then

autoradiographed to check for the removal of probe. The procedure was repeated if activity remained.

Stripped membranes were stored in sealed plastic bags at 4°C.

3.4.14. Analysis of Autoradiographs.

Autoradiographs produced from slot/dot blots, Northerns and <u>in vitro</u> translations were scanned on an Ultrascan XL™ Laser Densitometer (LKB).

The scanning data was stored on disc then analysed using the Gelscan XL™ Software Package (LKB). This package allowed the determination of the area under absorbance peaks displayed on the computer monitor. The baseline for the evaluation of the peak area can be selected by the programme in various ways using different criteria, or can be chosen manually to give a linear baseline. Manual selection was used where a single peak was to be assessed from several present on the scan, or where definite peaks could be seen and aligned across scans but due to the limitations of the parameters chosen they were ignored by the automatic baseline selection.

For both methods of baseline determination the integration programme in the package would then evaluate the peak area above the baseline. These peak area values were used for comparative purposes on samples off the same blot.

3.4.15. Statistical Analysis of Data.

Data was expressed as the mean plus or minus the standard error of the mean. Tests for significance between values obtained from the analysis of dot, slot and Northern blots as well as <u>in vitro</u> translations were made using a Students t-test (189). A significance level of 5% was adopted for all comparisons.

Chapter 4. Results

4.0. Preparation of Total RNA and its characterisation by in vitro translation.

The aim of the project described in this thesis was to examine whether the changes in activity of the calpain system components associated with β-agonist induced skeletal muscle hypertrophy (114) was also found in the expression of their respective mRNAs.

In order to achieve this aim developmental work had first to be carried out, which is described chronologically in the first two sections of this chapter.

This first section (section 4.0.) is concerned with the development of techniques for the isolation of total RNA from skeletal muscle and its subsequent characterization by in vitro translation.

The second section (section 4.1.) deals with the attempts to prepare specific probes for the detection of the mRNA for the components of the calpain system in the total RNA isolated from chicken and cattle skeletal muscle. For the calpains this initially involved the use of oligonucleotides then eventually cDNAs for the large subunits, and in the case of calpastatin a cDNA generated by the polymerase chain reaction.

Finally section 4.2. describes the use of these developed techniques and probes to quantify the specific mRNAs for actin, myosin light chain 2, calpain I and II large subunits as well as calpastatin in bovine muscle of animals treated with the growth promoting β-agonist cimaterol.

4.0.1. Extraction of Total RNA from Skeletal Muscle.

To analyse any changes in specific mRNA of the skeletal muscles of β-adrenergic agonist treated animals the total RNA used had to be intact as well as free from contaminant DNA and protein. However the extraction procedure had to be relatively quick so that samples from animal trials could be processed.

RNA extraction procedures are required to fulfil the following basic criteria:

1. Inhibit endogenous ribonucleases.

2. Deproteinise the RNA.

3. Physically separate the RNA from the other components in the homogenate.

There are two main types of RNA extraction procedures which differ in the protein denaturing agent that is used, namely phenol/chloroform or guanidinium salts.

For total RNA extraction experiments, and the bovine trial (section 4.2.2.), a phenol/chloroform method was used which developed into the final procedure as described in section 3.2.1.. During the development of this technique comparisons were made against a guanidinium thiocyanate based extraction procedure described in section 3.2.2. (166).

The phenol/chloroform method was modified from the procedure described by Clemens (164). The technique used two extraction buffers which had a high pH to allow poly(A)+ RNA to partition into the aqueous phase and inhibit RNAases present (164) as well as large volumes to dilute these degradative enzymes. At a pH below 5 the poly(A)+ RNA would be lost into the organic phenol/chloroform phase (165,193). The detergents (especially SDS) in the extraction solution mildly inhibited RNAases and promoted dissociation of the proteins from the RNA. The EDTA had the action of preventing aggregation of the RNA to each other and protein, by chelating the magnesium ions (165). Deproteinization of the homogenate was carried out by shaking it with an equal volume of phenol:chloroform 1:1 (v:v). The denatured protein was removed by centrifugation at high speed. The subsequent re-extraction of the phenol-chloroform and protein interface allowed the release of trapped RNA from the denatured protein.

In these initial experiments after the RNA was precipitated with ethanol (section 3.2.1.) it was differentially precipitated, i.e. 'cleaned', in a high salt concentration, 3M sodium acetate pH 6. This was reported to remove contaminant DNA, polysaccharides and small RNA, which all remained in solution whilst the RNA precipitated (164).

The guanidinium thiocyanate method used phenol/chloroform as well as the guanidinium salt in acidic conditions (section 3.2.2.) (166). This procedure was employed because of its relative speed compared with other guanidinium thiocyanate

methods, which often involved the use of caesium chloride 'cushions' (190). The guanidinium salts are suggested to be more effective than phenol/chloroform against tissues containing high levels of RNAase (191).

The homogenizing buffer (solution D) contained guanidinium thiocyanate which solubilised and denatured the protein, 2-mercaptoethanol that enhanced deproteinization by breaking disulphide bonds and sodium dodecyl sarcosinate which had the same effect as SDS (192). Extraction of the RNA was a one step process as described in section 3.2.2.. DNA and protein were reported to be removed in the interface and the organic phenol phase, whilst the aqueous phase contained the RNA which was precipitated with isopropanol. There were no high salt concentration cleaning steps in the method. Chomczynski and Sacchi (166) suggested that the isolated RNA was sufficiently clean to be used for Northern blot analysis and dot/slot blot hybridization.

Using the two methods outlined above total RNA was extracted from chicken breast muscle which was ground to a powder in liquid nitrogen so that the extractions were from a uniform source. Assessment of the quantity of nucleic acid and the protein associated with it was made on the total RNA extracted (section 3.2.5. and 3.2.12.). There was a larger quantity of nucleic acid isolated by the guanidinium thiocyanate based method than by phenol/chloroform, but the protein associated with it was also greater, Table 3.

Although there was more nucleic acid extracted by the guanidinium thiocyanate method it is impossible to distinguish between RNA and DNA using absorbance at 260nm as an assessment (section 3.2.5.). When a sample was subjected to electrophoresis on a 1% nondenaturing agarose gel (Figure 13) it was apparent that the high absorbance of the 'RNA' prepared by the guanidinium method was due to a high content of DNA which was clearly visible as large amount of the low mobility DNA band and smear compared with the 28S and 18S rRNA bands in the samples.

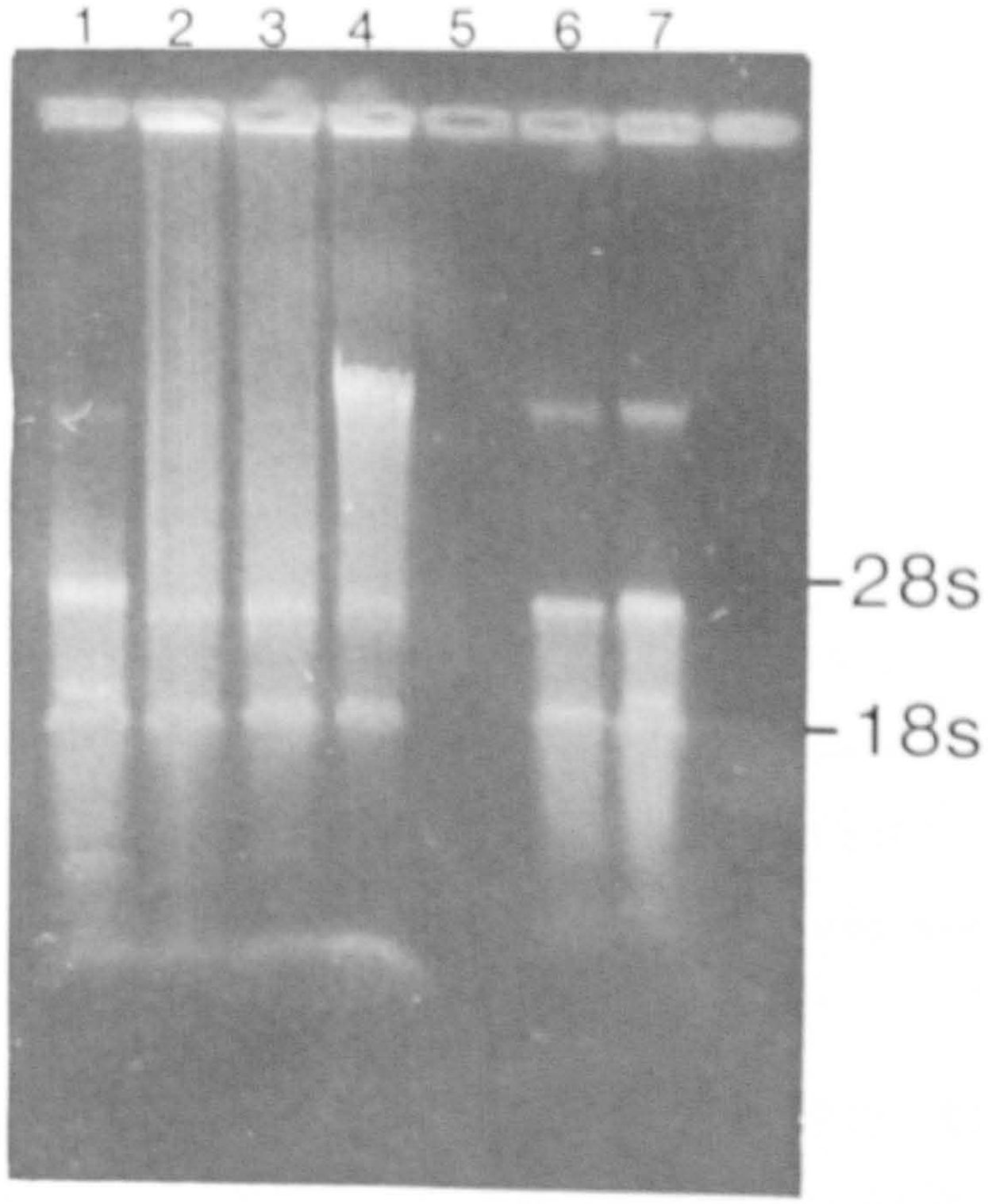
The phenol/chloroform method contains steps that are purposely included for removing DNA after the RNA has been precipitated. High salt cleaning of the extracted RNA appears to be necessary to remove DNA carried over from the extraction procedure.

Table 3. Comparison of total RNA extraction procedures. Total RNA was extracted from chicken skeletal muscle using a phenol/chloroform or guanidinium thiocyanate method, as outlined in the text.

	Extraction method	
	Phenol Chloroform (n=3)	Guanidinium Thiocyanate (n=3)
Nucleic acid (µg/g muscle)	266 ± 28	780 ± 61
Protein (µg/g muscle)	9.6 ± 0.8	91 ± 7.2
Nucleic acid : Protein (ratio per g muscle)	28 : 1	9 : 1

(values are the mean ± SEM)

Figure 13: Nondenaturing 1% agarose gel electrophoresis of total RNA prepared and 'cleaned' by different procedures. Lanes 1-4 guanidinium thiocyanate prepared total RNA with various 'cleaning steps': lane 1 two 3M sodium acetate pH6 cleaning steps, lane 2 two 3M sodium acetate pH6 plus two 4M lithium chloride steps, lane 3 two 4M lithium chloride steps and lane 4 no cleaning steps. Lanes 6 and 7 are RNA prepared by phenol/chloroform and 3M sodium acetate pH6 reprecipitation. Each lane was loaded with approximately the same quantity of nucleic acid (3µg).



Removal of DNA from RNA samples.

As the guanidinium prepared sample shown in lane 4 of Figure 13 had large quantities of DNA present it was used to determine a method of removing DNA from RNA samples. High salt concentrations differentially precipitate RNA whilst leaving the DNA and small RNAs in solution (164,165). The results of high salt 'cleaning' are shown in Figure 13. Both sodium acetate and lithium chloride high salt precipitation procedures qualitatively reduce the amount of DNA present in the RNA samples. Lithium chloride was the most effective method (described in section 3.2.1.). The two salts appear to have specific effects, solubilising and precipitating different sizes of nucleic acids.

To compare the effect on the yield of RNA of the cleaning steps on the same muscle source, RNA was extracted using phenol/chloroform then cleaned with the two different methods. The results of the extractions, are shown in Table 4. There was a reduced yield when sodium acetate and lithium chloride were used compared with sodium acetate alone. When the samples were subjected to electrophoresis it could be seen, by comparing the relative intensities of the RNA to the large DNA band, that there was less DNA in the samples cleaned using sodium acetate and lithium chloride, Figure 14. The reduced yield was probably due to the removal of the DNA remaining in the samples cleaned with sodium acetate alone.

Further qualitative assessments on the effectiveness of the two salts are indicated in Figure 15. The large DNA fragment in the total RNA remains after 3M sodium acetate washing which is virtually completely removed by subsequent 4M lithium chloride precipitation. With bovine samples it was later found that two 4M lithium chloride cleaning steps could remove all the DNA present as assessed by nondenaturing agarose gel electrophoresis, Figure 16. This was the method adopted for all subsequent extractions and cleaning of total RNA, including the bovine trial (section 4.2.), as described in the methods (section 3.2.1.). By eliminating the 3M sodium acetate step the possible losses caused by reprecipitation and sample handling were decreased.

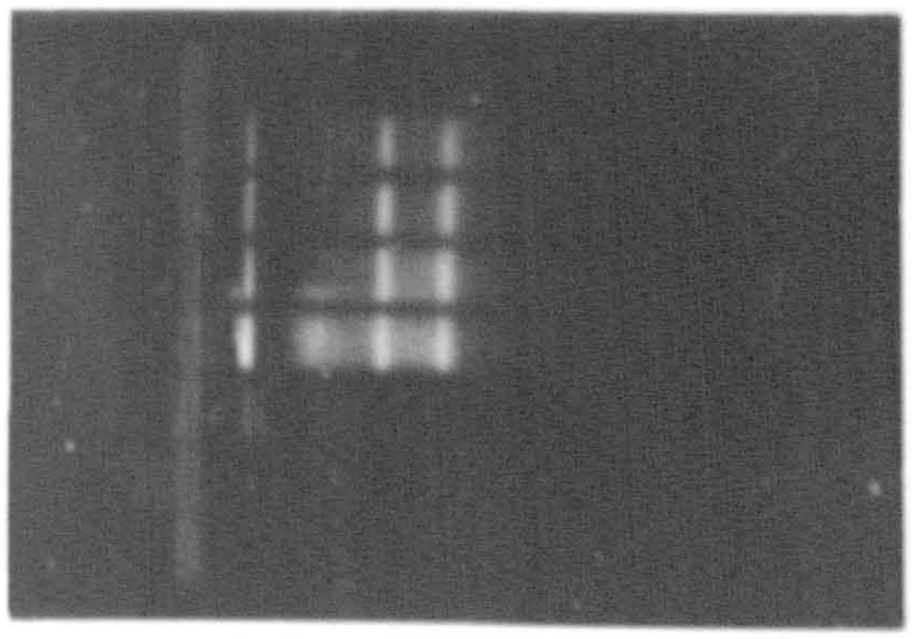
Table 4. The effect of RNA high salt reprecipitation ('cleaning') on the yield of nucleic acid. Total RNA was extracted from the same chicken breast muscle source (2g per extraction) by the phenol/chloroform method. The extracted nucleic acid was then cleaned by two different subsequent cleaning procedures:
A, cleaning twice with 3M sodium acetate pH6.
B, cleaning once with 3M sodium acetate pH6 then once with 4M lithium chloride.
Nucleic acid concentration was determined by absorbance at 260nm.

	Extraction Procedure	
	A (n=4)	B (n=4)
Nucleic acid (µg/g muscle)	470 ± 21	359 ± 19

(Values are the mean ± SEM)

Figure 14: Nondenaturing 1% agarose gel electrophoresis of total RNA from chicken skeletal muscle prepared by the phenol/chloroform extraction 'cleaned' by different procedures. Gel A; cleaning twice with 3M sodium acetate pH6 (n=4). Gel B; reprecipitation once with 3M sodium acetate pH6 then 4M lithium chloride (n=4). Each lane was loaded with approximately the same quantity of nucleic acid (2-3µg).

A



B

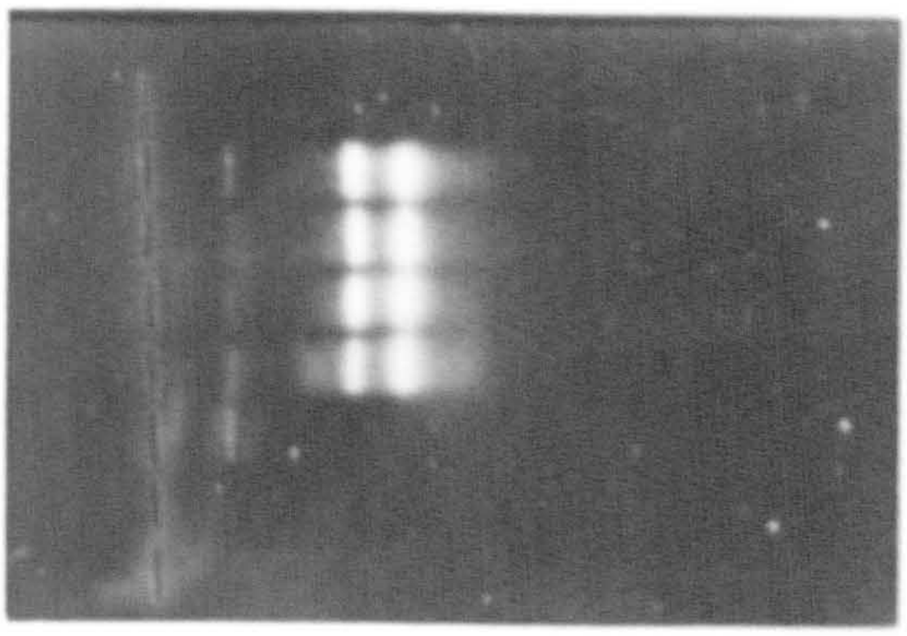


Figure 15: Nondenaturing 1% agarose gel electrophoresis of total RNA prepared by phenol/chloroform extraction from bovine skeletal muscle and 'cleaned' by different procedures. Lanes 1-4 samples 'cleaned' twice with 3M sodium acteate pH6. Lanes 5-8 samples from lanes 1-4 reprecipitated twice with 4M lithium chloride. Each lane was loaded with approximately 2µg of nucleic acid.

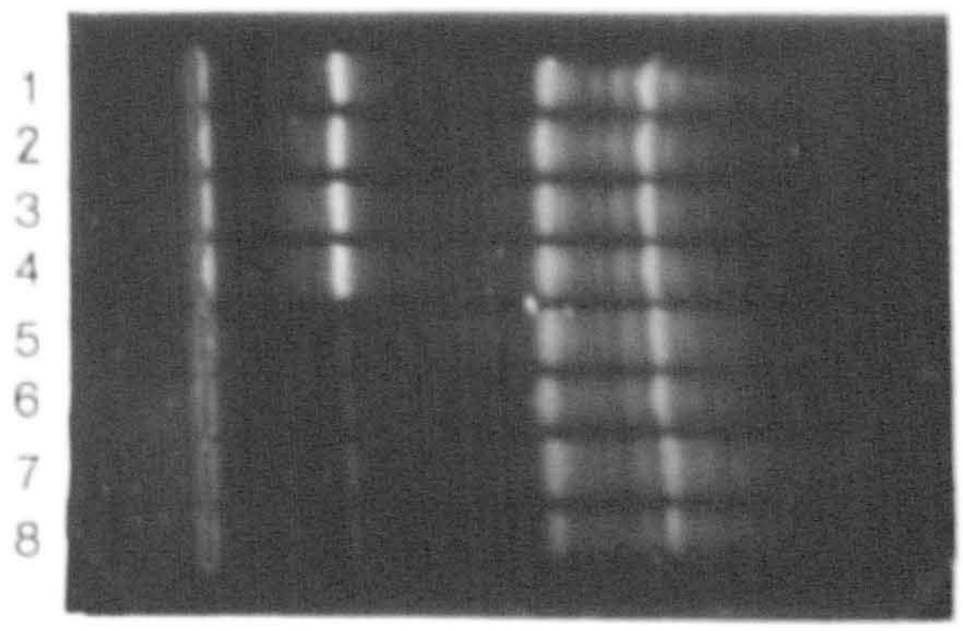
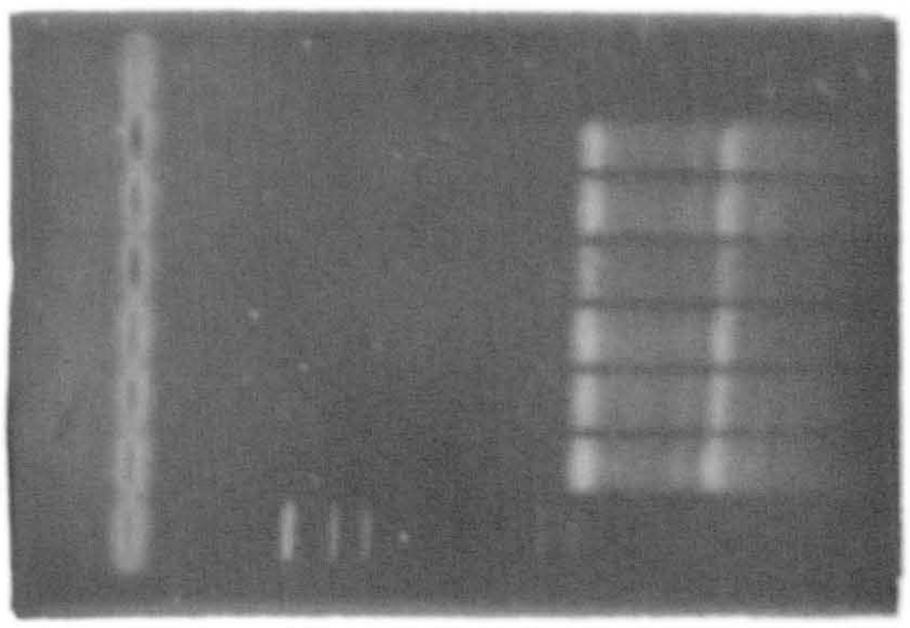


Figure 16: Nondenaturing 1% agarose gel analysis of total RNA extracted from bovine skeletal muscle using phenol/ chloroform and two 4M lithium chloride steps. Each lane was loaded with approximately 2µg of nucleic acid.



Although phenol/chloroform is not as strong a denaturing agent as guanidinium salts it appeared to be competent in producing RNA which was intact and free of protein (192). The effectiveness of the phenol/chloroform method was enhanced by the use of high salt precipitation of the RNA. Extraction of RNA from skeletal muscle encounters different problems than that of extraction from tissues such as liver. In skeletal muscle there is large quantity of protein which is physically removed as interface material in the phenol/chloroform extraction. Guanidinium salt methods are suited to tissues in which there are relatively high levels of RNases such as the pancreas where this technique has been effective in producing intact RNA (190). There appeared to be no advantage in using the guanidinium thiocyanate method for extracting RNA from skeletal muscle.

4.0.2. Extraction of Total RNA from Skeletal Muscle of Various Species.

Skeletal muscle total RNA for Northern blot analysis was extracted using the basic phenol/chloroform procedure (section 3.2.1.) then cleaned with the two high salt reprecipitation methods. The quantity of nucleic acid resulting from the extraction after each of the two subsequent 'cleaning' steps is shown in Table 5. The effect of 3M sodium acetate pH6 followed by 4M lithium chloride washing on the bovine skeletal muscle RNA samples can been seen in Figure 15.

The content of total RNA in lamb and bovine samples is virtually the same. Chicken muscle contains considerably more total RNA than the other samples. This is probably due to the extraction being from young birds, approximately 3 weeks old, which are growing rapidly. The data on the effects of cleaning the RNA shows the loss of nucleic acid with differential precipitation 'cleaning'; some of the loss was due to the removal of contaminant DNA but also because of the repeated precipitation.

Table 5. Total RNA extraction from skeletal muscle of various species using phenol/chloroform followed by two different subsequent high salt reprecipitations, first with two 3M sodium acetate pH6 reprecipitations then two 4M lithium chloride steps. The nucleic acid present was assessed by absorbance at 260nm. The percentage yield of the nucleic acid after lithium chloride treatment of the sodium acetate cleaned RNA pellet is also shown.

Source of muscle	Nucleic acid present after Cleaning procedure (µg/g muscle)		Yield of Nucleic acid between cleaning steps
	3M sodium acetate	4M lithium chloride	
Lamb (L.dorsi)	171 ± 10	119 ± 7	68%
Bovine 1 (L.dorsi)	167 ± 10	107 ± 9	64%
Bovine 2 (L.dorsi)	152 ± 2	109 ± 9	72%
Chicken • (Pectoralis)	470 ± 21 [1]	359 ± 19 [2]	76%

• These samples were from two separate preparations of the same muscle powder stock; [1] was cleaned twice with 3M sodium acetate, [2] was cleaned once with 3M sodium acetate followed by one precipitation with 4M lithium chloride.

4.0.3. In Vitro Translation of mRNA by a Rabbit Reticulocyte Lysate System.

In vitro translation was used to determine whether the RNA extracted was intact by its ability to produce proteins monitored with L-[^{35}S]-methionine incorporation. This was detected as TCA precipitable protein and/or autoradiographs of the translation products on SDS-PAGE gels (section 3.2.10. and 3.2.11.).

Initial translations were carried out on chicken skeletal muscle total RNA using the reticulocyte lysate provided by the John Innes Research Institute (section 3.2.10) in a translation cocktail, developed by the Plant Physiology section within the Faculty, optimized for the translation of plant total RNA. The system contained potassium ions at 112mM, magnesium ions at 3.4mM, which are critical in the in vitro translation, and the lysate at 66% (v/v). The total RNA gave no detectable incorporation of ^{35}S into TCA precipitable protein above the background blank and no translation products specific to the muscle total RNA when the samples were analysed on a 10% SDS-PAGE. Beta-globulin mRNA (Boehringer) also failed to give any ^{35}S incorporation into protein so it was assumed that the lysate mix was ineffective.

For all the translations using the system there was a high level of blank activity in the TCA precipitated ^{35}S labelled proteins from the translation mix (section 3.2.10.). Typically blank samples with no RNA present had incorporation values of 41×10^3 cpm/2µl compared with 36×10^3 cpm/2µl when 4µg of total RNA was present. This level of incorporation was the same in all the translations, the blank was slightly above or below that of the rest of the samples. Normally a five fold greater incorporation was expected to be produced from total RNA above the blank.

As the reticulocyte lysate system was suspected of not being capable of in vitro translation it was changed to another which had been optimized for Tobacco Mosaic Virus (TMV) mRNA (Amersham). This had optimum ion concentrations of 178mM potassium, 1.6mM magnesium, the optimum range being 100-135mM and 2.5-3.5mM respectively (176), and lysate at 64% (v/v). Using this system, which was employed essentially in the same manner as described in section 3.2.10., on chicken skeletal muscle

total RNA it produced similar results to the John Innes lysate, that is no incorporation of ^{35}S in the test samples above the blank.

Using a fresh batch of John Innes lysate the translation cocktail was modified to include the antibiotic chloramphenicol, at 10µg/ml, to inhibit bacterial growth in the lysate during the incubation period. The RNA was introduced to the translation mix as a pellet to allow the reticulocyte lysate to be at a higher concentration, 71% (v/v) and the magnesium and potassium ions were at 2.1mM and 105mM respectively. The in vitro translation was carried out essentially as described in section 3.2.10.. This method produced the greatest incorporation of ^{35}S in the test samples of chicken muscle poly(A)+ RNA that had been seen so far, shown inTable 6. There was also a reduction in the background, the TCA precipitable products of the blank, to below $5x10^3$ cpm/2µl lysate.

Although there was ^{35}S incorporation into TCA precipitable protein, it was not seen in all the samples. The ^{35}S protein products could not be evaluated from SDS-PAGE because of technical difficulties in producing dried gels of sufficient quality. However improved translation appeared to have taken place, probably caused by inhibition of bacterial growth in the lysate.

Greater incorporation was achieved when the RNA was added to the lysate mix as a solution as described in section 3.2.10 and the type of L-^{35}S-methionine used was altered to an 'in vivo labelling grade' type isotope (Amersham). In changing the translation mix the potassium and magnesium ion concentrations were modified to those in section 3.2.10., but this was not a substantial alteration. Although an autoradiograph of the translation products was not produced due to poor gel drying it appeared that the translation cocktail provided the appropriate conditions for effective in vitro translation, shown in Table 7. These conditions were adopted to give the finalized method, described in section 3.2.10, and used for the subsequent translations of the bovine trial total RNA samples (section 4.2.3.).

Table 6. Assessment of incorporation of L-[^{35}S]-methionine into TCA precipitable protein after *in vitro* translation of chicken liver total RNA and skeletal muscle poly(A)+ RNA.

RNA Sample	RNA (μg)	Incorporation of ^{35}S cpm/2μl lysate	Incorporation over background
Blank	-	3548	-
Chicken muscle (poly(A)+)	0.4	6931	95%
Liver (Total)	10.0	6735	90%

Table 7. Assessment of incorporation of L-[^{35}S]-methionine into TCA precipitable protein after the *in vitro* translation of chicken skeletal muscle total RNA.

Quantity of Total RNA (μg)	Incorporation of ^{35}S cpm/2μl lysate	Incorporation over background
0	3360	-
3.2	14588	334 %
10.0	7295	117 %
20.0	4943	47 %

4.1. The Selection, Generation and Use of DNA Hybridisation Probes for the Detection of the Calpain System's mRNAs.

The aim of the project was to examine any changes in the gene expression of the components of the calpain system detected by alterations in the quantity of mRNA specific for the calpain isoforms and calpastatin when various species, in particular farm species, were treated with β-adrenergic agonists.

The cDNAs or other complementary nucleotide sequences to the calpain and calpastatin mRNAs were required as probes for Northern analysis and/or slot blot hybridization to quantify any changes in their mRNA. In order to quantify changes in the gene expression of the calpain isoforms probes to the calpain large subunit were required. The small subunits are identical in calpain I and II so they were not suitable as calpain isoform specific probes (22).

Initially work concentrated on the development of calpain mRNA probes as there was cDNA sequence information available but little on calpastatin. Subsequently sequences for the inhibitor were published which allowed the generation of a calpastatin cDNA by PCR (section 4.1.5.).

4.1.1 The Use of an Oligonucleotide as a Hybridization Probe to Calpain Large Subunit mRNA; Oligonucleotide I.

At the time of commencing the project several cDNAs to the large subunit of calpain had been sequenced and published in the literature; they included:

1) Chicken calpain large subunit cDNA which included the complete amino acid coding sequence (14).

2) Rabbit calpain large subunit cDNAs to both calpain I and II which were only for part of the amino acid coding sequence (15).

3) Human calpain I large subunit cDNA which was for the entire amino acid coding sequence (16) and at a later stage the cDNA to the complete large subunit of calpain II (17).

It proved impossible to obtain calpain cDNAs from the laboratories where they had originally be isolated, therefore an oligonucleotide was used as a probe to the calpain

large subunit mRNA. Oligonucleotides have been used as hybridization probes for the isolation of cloned genes and cDNA (194,195) and subsequently on Northern blots (196).

It was decided to make a complementary oligonucleotide to part of the chicken calpain large subunit cDNA. This sequence was chosen for several reasons:

1) A β-agonist chicken trial was being carried out within the Faculty.

2) The calpain system in chicken skeletal muscle was suggested to contain only one isoform of calpain identified on Northern blots and by ion exchange chromatography (14,12). Therefore it was a relatively simple system to study compared with mammalian muscle where both isoforms had been isolated (15,16,17).

3) Chicken skeletal muscle calpain had been classified as a calpain II-like isoform (12). This was the enzyme activity that changed significantly in the skeletal muscle of lambs in a β-adrenergic agonist trial carried out in our laboratories (114).

Although the calpain system in chicken skeletal muscle had not been analysed for changes in activity on treatment with β-adrenergic agonists, chicken skeletal muscle had been reported to respond to their growth promoting effects (197).

The isoforms of the calpain large subunit have a high degree of homology at the amino acid (55-70%) and nucleotide level (60-80%). The cDNAs available in the literature were aligned to find the regions, particularly in calpain II, which had a high degree of homology to chicken calpain cDNA, in order to identify a sequence suitable as an oligonucleotide probe. Initially the rabbit isoforms were used for comparison. It was envisaged that homology would be advantageous if the oligonucleotide was to be used to probe mRNA from other species.

The oligonucleotide was selected from domain III of the enzyme as domain II and IV have homology to thiol proteinase and calmodulin-like calcium binding proteins respectively (14). Domain I was not selected, although this appears to be different in the two isoforms (17) and possibly gives the enzyme its calcium sensitivity, as the rabbit cDNAs did not contain any sequence for domain I. A 'best-fit' oligonucleotide sequence was not made as the initial aim was to probe total RNA isolated from a chicken muscle.

The selected sequence, oligonucleotide I, is shown in Figure 17 along with the corresponding region of rabbit and human calpain large subunit isoforms. Table 8 shows the homology between the chicken cDNA oligonucleotide sequence and the other sequences. The position of the oligonucleotide in the cDNAs which have been isolated is shown in Figure 18

To be used as a hybridization probe the chicken sequence was made as antisense and called oligonucleotide I;

5' AAA GGT GGA GGG CAC AAC GAT GTA CTC GCC AGG GGG CAG 3'

The oligonucleotide I has a high GC to AT ratio, 24:15, thereby increasing the hybridization stability to the complementary sequence.

Figure 17. The chicken calpain large subunit sequence chosen for oligonucleotide I aligned to the corresponding cDNA sequences of other species. Numbers in brackets indicate the position of the oligonucleotide region in the cDNA. The oligonucleotide I is complementary to that of the cDNA.

chicken calpain cDNA (1437-1475)

5' CTG CCC CCT GGC GAG TAC ATC GTT GTG CCC TCC ACC TTT 3'

rabbit calpain I cDNA (229-267)

5' CTG CCG CCC GGG GAG TAC GTG GTG GTG CCC TCG ACC TTC 3'

rabbit calpain II cDNA (595-633)

5' CTG CCG CCG GGG GAG TAC ATC CTG GTG CCC TCC ACC TTC 3'

human calpain I cDNA (1465-1503)

5' CTG CCA CCC GGG GAG TAT GTG GTG GTG CCC TCC ACC TTC 3'

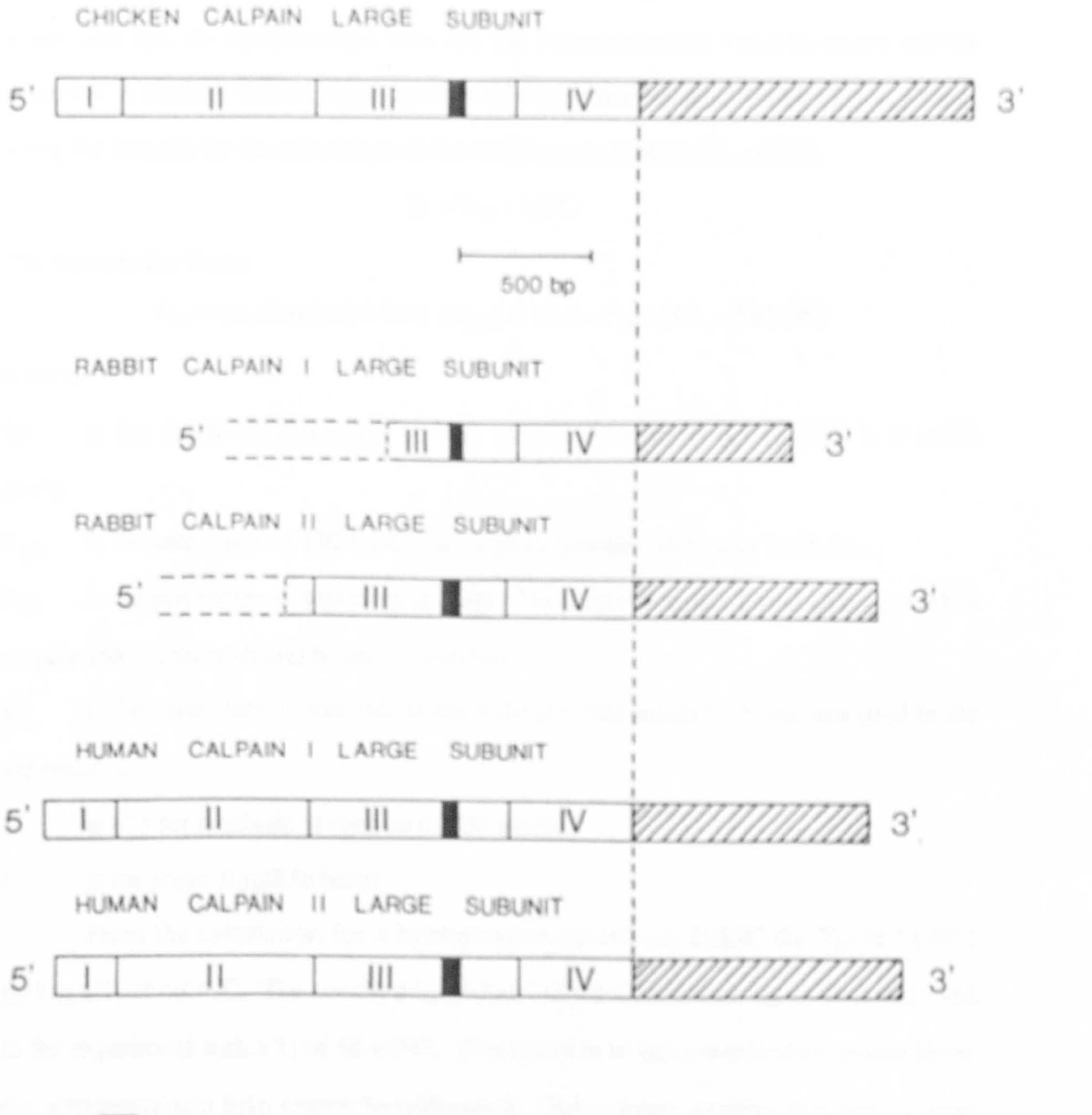
human calpain II cDNA (1429-1467)

5' CTG CCG CCA GGA GAG TAC ATT CTC GTG CCT TCC ACC TTC 3'

Table 8. Percentage homology of the sequence selected from chicken calpain large subunit cDNA for oligonucleotide I with other calpain large subunit cDNAs.

	Homology of oligonucleotide sequence			
	rabbit calpain I	rabbit calpain II	human calpain I	human calpain II
chicken calpain	79%	85%	79%	79%

Figure 18: The relative position of oligonucleotide I in the cDNAs of the other calpain large subunit isoforms from different species (15,16,17). The sequences are aligned at the 3' coding/noncoding junction. The rabbit sequences were published incomplete (15).



The oligonucleotide I was labelled using polynucleotide kinase and [γ-^{32}P] ATP, by which the 5'hydroxyl group of the oligonucleotide had a phosphate group added (section 3.3.2.). Estimation of isotope incorporation was made using denaturing polyacrylamide-urea gel electrophoresis (section 3.4.11. and 4.1.3.). In order to achieve stable and specific hybridization between the oligonucleotide I and its target mRNA sequence in chicken mRNA, an estimation of the incubation temperature (T_i) was made using the formula for the calculation of the melting temperature (T_m) (198);

$$T_i = T_m - 15^oC$$

The formula for Tm is;

$$T_m = 16.6 \log[M] + 0.41 [P_{gc}] + 81.5 - P_m - B/L - 0.65 [P_f]$$

where,

M is the molar concentration of Na+, to a maximum of 0.5 (1xSSC is 0.165M Na^+).

P_{gc} is the percentage of GC bases. In oligonucleotide I this value is 61.5%.

P_m is the percentage mismatches in bases. For oligonucleotide I used against chicken calpain mRNA there should be no mismatches.

P_f is the percentage formamide in the hybridization solution. None was used in the experiment.

B is 675 for synthetic probes up to 100 bases.

L is the probe length in bases.

From the calculation for a hybridization containing 2xSSC the T_m is 81.4^{o}C giving a T_i of 66.4^{o}C. The standard hybridization solution containing 6xSSC was used in the experiment with a T_i of 66-68^{o}C. This increase in salt concentration would lower the stringency and help ensure hybridization. Subsequent washing procedures were carried out with 2xSSC and 0.1% (w/v) SDS at the same temperature.

The oligonucleotide I end-labelled probe was first used on a Northern blot of chicken skeletal muscle and liver total and poly(A)+ RNA, Figure 19. There was clear hybridization to a single band in the poly(A)+ RNA from muscle with none to total RNA.

Figure 19: Northern blot of chicken skeletal muscle and liver total RNA and Poly(A)+ RNA probed with 5' end-labelled oligonucleotide I. Hybridization and washing conditions were carried out as described in the text. The positions of the 28S and 18S ribosomal RNAs are indicated.

Lane No.	Sample
1	1µg Hind III lambda DNA markers.
2	5µg poly(A)+ RNA, chicken skeletal muscle.
3	4µg poly(A)+ RNA, chicken liver.
4	15µg total RNA, chicken liver.
5	20µg total RNA, chicken liver.
6	empty
7	15µg total RNA, chicken skeletal muscle.

The size of the calpain large subunit mRNA was approximately 3.5 kb, in agreement with other research groups (14-17).

Chicken calpain large subunit mRNA is a low copy number species at picogram amounts per microgram total RNA, for example 60pg/50µg skeletal muscle total RNA (199). This may explain the lack of hybridization to the calpain mRNA in the total RNA loaded. The poly(A)+ RNA had been prepared by double passage through oligo-d(T) cellulose and approximately 5µg had been loaded (section 3.2.3.), so was an enriched source of calpain mRNA. Liver has the lowest quantity of calpain large subunit mRNA, less than half that of skeletal muscle, but the lack of a hybridization signal in the liver poly(A)+ RNA was probably due to its poor quality.

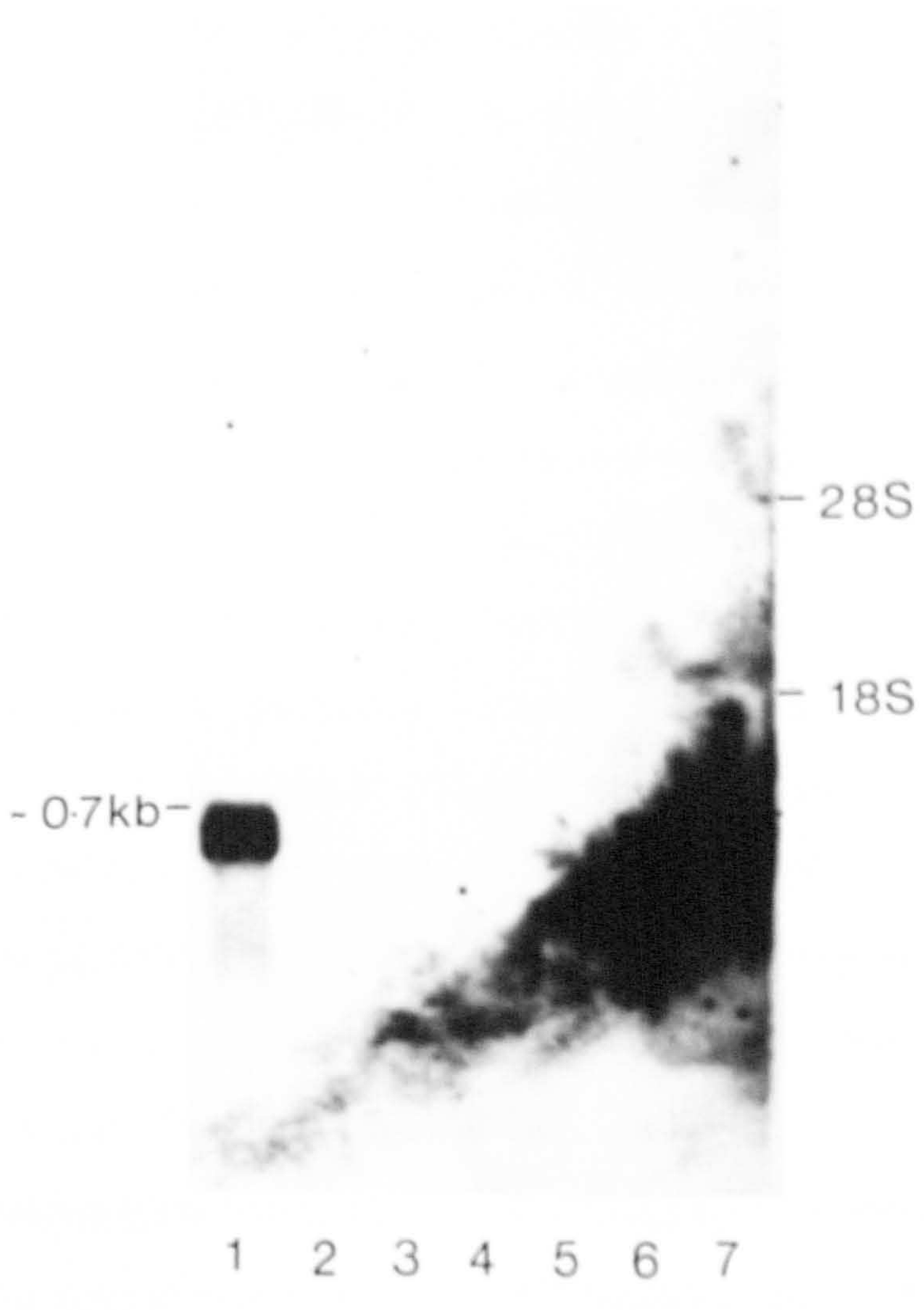
The meagre quantity of calpain mRNA in muscle could be seen by comparison when the Northern blot was reprobed with chicken myosin light chain 2 (MLC2) cDNA, Figure 20. The chicken MLC2 probe is described in appendix A. The membrane was hybridized at 55°C then washed at increasing stringency to 65°C with 0.1xSSC and 0.1% (w/v) SDS. It can be seen that the strength of the hybridization signal is much greater than that for calpain which was washed at slightly lower stringency, although the the hybridization bands in the muscle total RNA are obscured by nonspecific background. MLC2 mRNA transcripts are expressed in a higher copy number than calpain in skeletal muscle total RNA.

In subsequent experiments in order to detect calpain large subunit mRNA in skeletal muscle total RNA Northern blots up to 50µg was used. Total RNA was favoured because it would be relatively easier to handle and prepare than poly(A)+ RNA, especially when dealing with samples from an animal trial.

The use of the oligonucleotide I probe across species.

At this point in the project the chicken trials with β-adrenergic agonist were not producing a predicted growth response. There were no changes in the activity of calpain and calpastatin in skeletal muscles examined, although there had been reported growth effects in β-agonist-treated poultry described in the literature (197). A bovine β-agonist trial was being carried out within the department using the β-adrenergic agonist cimaterol.

Figure 20: Northern blot of Figure 19 reprobed with chicken myosin light chain 2 (MLC2) cDNA. The conditions are outlined in the text. The positions of the 18S and 28S ribosomal RNAs are indicated.



Lane No.	Sample
1	5µg poly(A)+ RNA, chicken skeletal muscle.
2	4µg poly(A)+ RNA, chicken liver.
3	15µg total RNA, chicken liver.
4	20µg total RNA, chicken liver.
5	empty
6	15µg total RNA, chicken skeletal muscle.
7	20µg total RNA, chicken skeletal muscle.

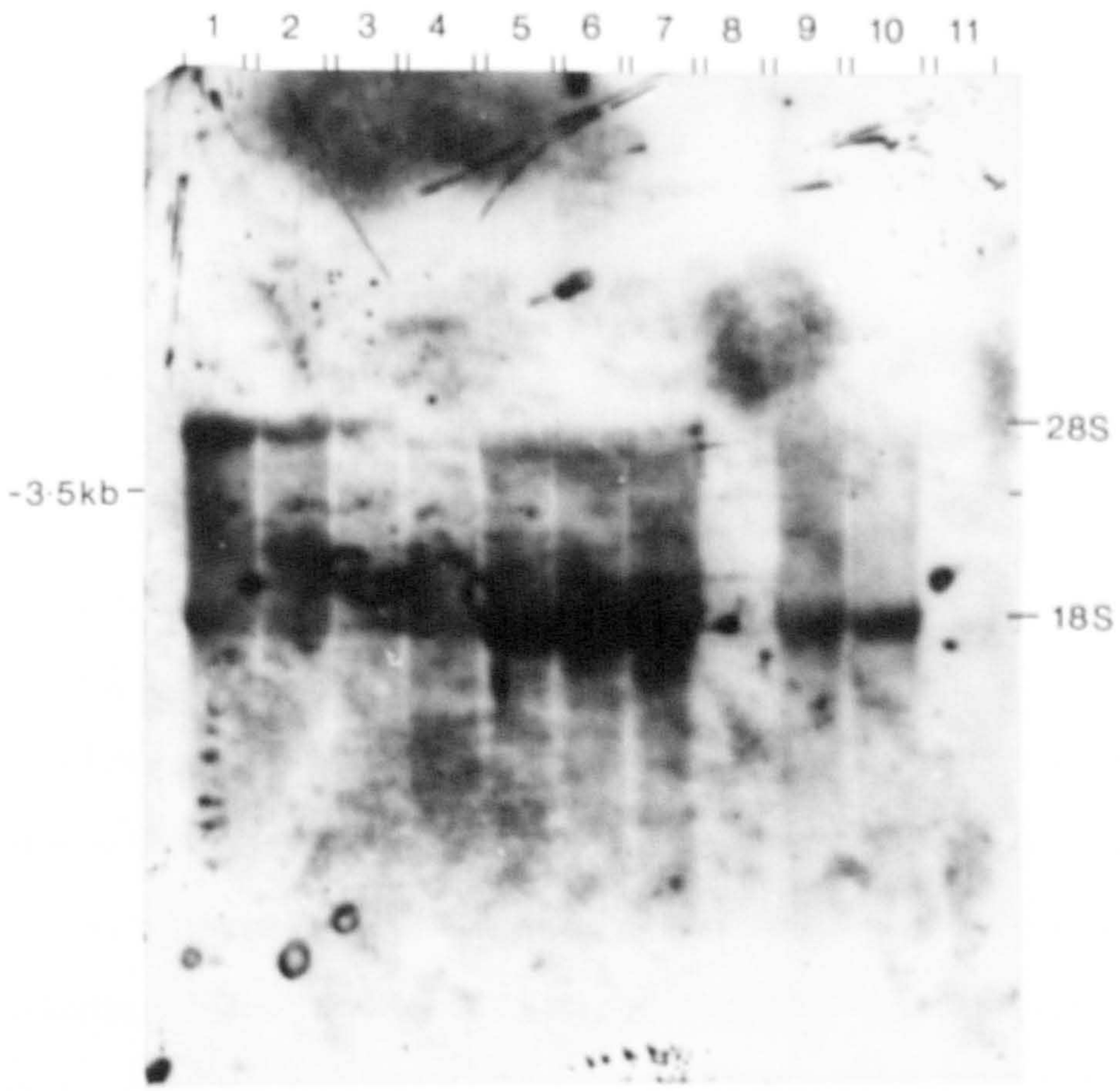
Beta-agonist induced skeletal muscle hypertrophy had been observed in bovine species by other research groups (118,200) and in experiments carried out within our laboratories (unpublished observations). Also the calpain and calpastatin activities were known to respond to β-adrenergic agonists (unreported observation) similar to those seen in lambs (114). Therefore attention was focused on detecting calpain large subunit mRNA in the skeletal muscles of this species.

The oligonucleotide I probe had been made to have high homology to the calpain large subunit cDNAs of other species, therefore it was possible that it would detect bovine calpain large subunit mRNA at low stringency probing and membrane washing. A decrease in the stringency is necessary as the stability of duplex of the two strands is reduced as the number of mismatches increases. As the bovine sequence was expected to have approximately 20-25% mismatch to the oligonucleotide (Table 8) the formula used to calculate the hybridization temperature was employed assuming that the mismatch was 20% and the hybridization solution contained 2xSSC. The value obtained for T_m was 56°C. A 15°C reduction in this temperature for T_i would have resulted in the possible precipitation of the BSA and SDS in the hybridization solution. In order to elevate the temperature the salt concentration of the hybridization solution was increased to 6xSSC (the standard hybridization salt concentration) which allowed T_i to be raised to 50-55°C. Although the T_m equation does not hold at these salt concentrations (6xSSC) it does give an indication of the temperature and hybridization solution composition which should be used for the degree of mismatching expected.

By carrying out hybridization at low stringency then subsequently washing the membrane in steps of increased stringency (decreased salt and increased temperature), with the analysis of the results of each wash, identification of calpain large subunit mRNA hybridization signals across species would hopefully be achieved.

A Northern blot was made of bovine and chicken total RNA, up to 100µg, and poly(A)+ enriched RNA, prepared by a single passage through a oligo-d(T) (3.2.3.). The Northern blot was probed with oligonucleotide I (30pmol) labelled with 20pmol of [γ-^{32}P] ATP (>5000 Ci/mmol) using polynucleotide kinase in low stringency

Figure 21: Northern blot of chicken and bovine total and poly(A)+ RNA probed with the 5'end-labelled oligonucleotide I. The conditions are outlined in the text. The locations of the 28S and 18S ribosomal RNAs are indicated.



Lane No.	Sample
1	50µg total RNA, bovine skeletal muscle.
2	25µg total RNA, bovine skeletal muscle.
3	6µg poly(A)+ RNA, bovine skeletal muscle.
4	6µg poly(A)+ RNA, chicken skeletal muscle.
5	30µg total RNA, chicken skeletal muscle.
6	50µg total RNA, chicken skeletal muscle.
7	100µg total RNA, chicken skeletal muscle.
8	6µg poly(A)+ RNA, chicken skeletal muscle.
9	50µg total RNA, chicken liver.
10	25µg total RNA, chicken liver.
11	1µg Hind III lambda DNA markers.

hybridization conditions (50°C) (section 3.4.12.). The membrane was washed to 55°C with 6xSSC to give the autoradiograph shown in Figure 21.

The oligonucleotide I did not pick up a specific hybridization band for calpain large subunit in chicken or bovine RNA; however the signal may have been obscured by the high background probably caused by lowering the hybridization stringency. There was a weak hybridization signal visible at the proposed position of the chicken calpain large subunit mRNA at approximately 3.5kb which can just be seen in the bovine RNA samples (Figure 21). There was no double band detectable in the bovine sample indicating hybridization to the two large subunit isoforms mRNAs. However, they were reported to be of similar size in rabbit and human (15-17).

The Effectiveness of MLC2 and α-actin cDNAs as hybridization probes across species compared with oligonucleotide I.

To check the poly(A)+ enriched and total RNA had not been degraded during extraction the membranes were stripped of the oligonucleotide I probe (section 3.4.13.), then reprobed.

Figure 22 shows the membrane probed with nick translation-labelled chicken MLC2 cDNA at 55°C then washed to 2xSSC plus 0.1% (w/v) SDS at 65°C. The probe hybridized strongly to chicken muscle RNA, at the expected size of 0.7kb, and this was not lost on higher stringency washing. There was high nonspecific hybridization within the chicken total and poly(A)+ RNA samples, but this was removed at higher stringency washing unlike the background seen in the oligonucleotide I probed Northern blot (Figure 21). The autoradiograph shown in Figure 22 was also selected to illustrate that the chicken MLC2 cDNA could hybridize to the bovine muscle total and poly(A)+ RNA, although this was not as strong as that to the chicken RNA, unlike the α-actin cDNA (see below).

Figure 23 is the membrane of Figure 22 reprobed with the nick translation labelled mouse α-actin cDNA. The mouse α-actin probe is described in appendix A. The Northern blot was hybridized at 55°C and washed with 2xSSC plus 0.1% (w/v) SDS at 65°C. The mouse α-actin cDNA binds to both chicken and bovine α-actin

Figure 22: Northern blot of figure 21 reprobed with chicken MLC2 cDNA. The conditions are described in the text. The samples in the lanes are indicated in figure 21. The positions of the 28S and 18S ribosomal RNAs are marked.

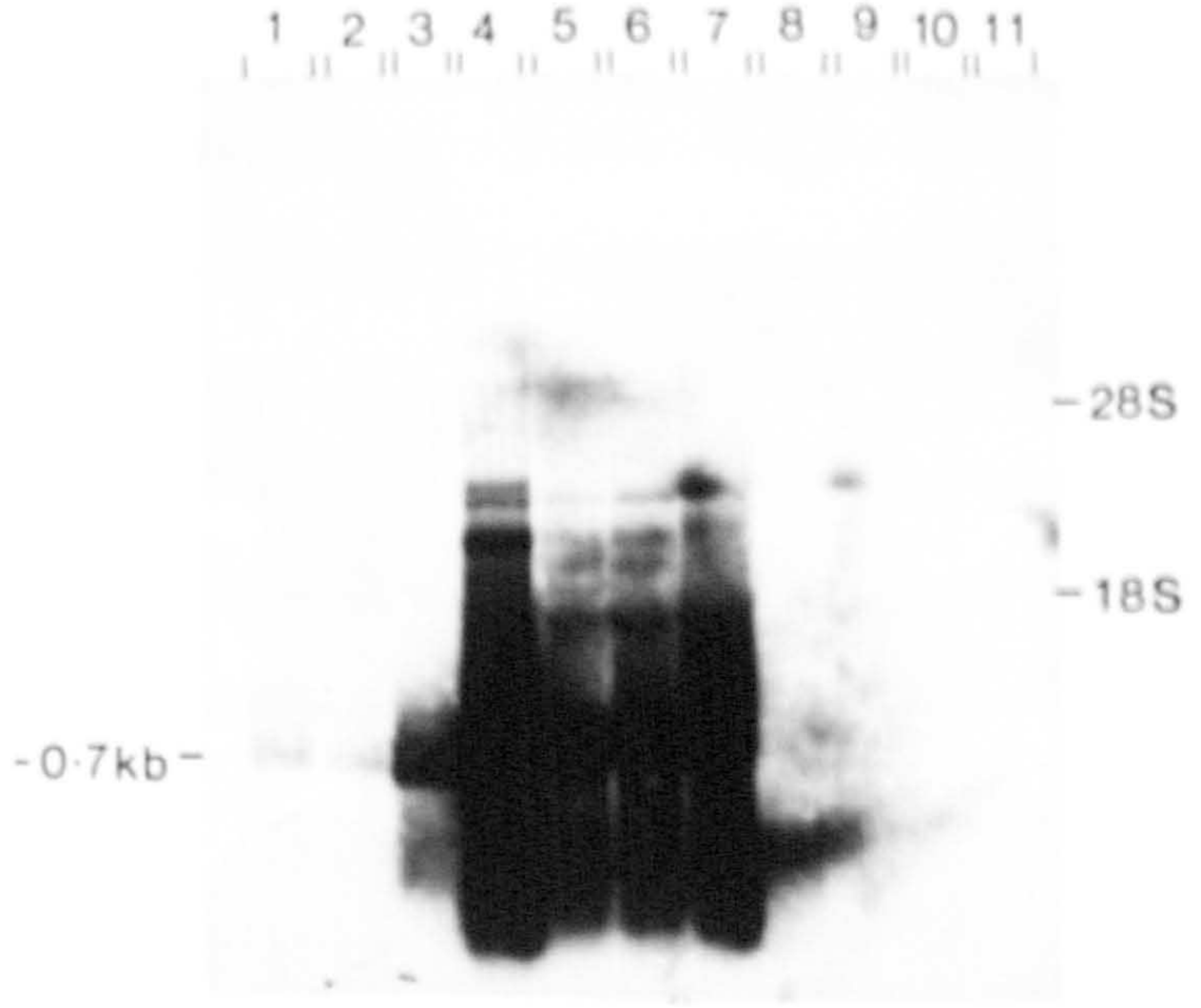
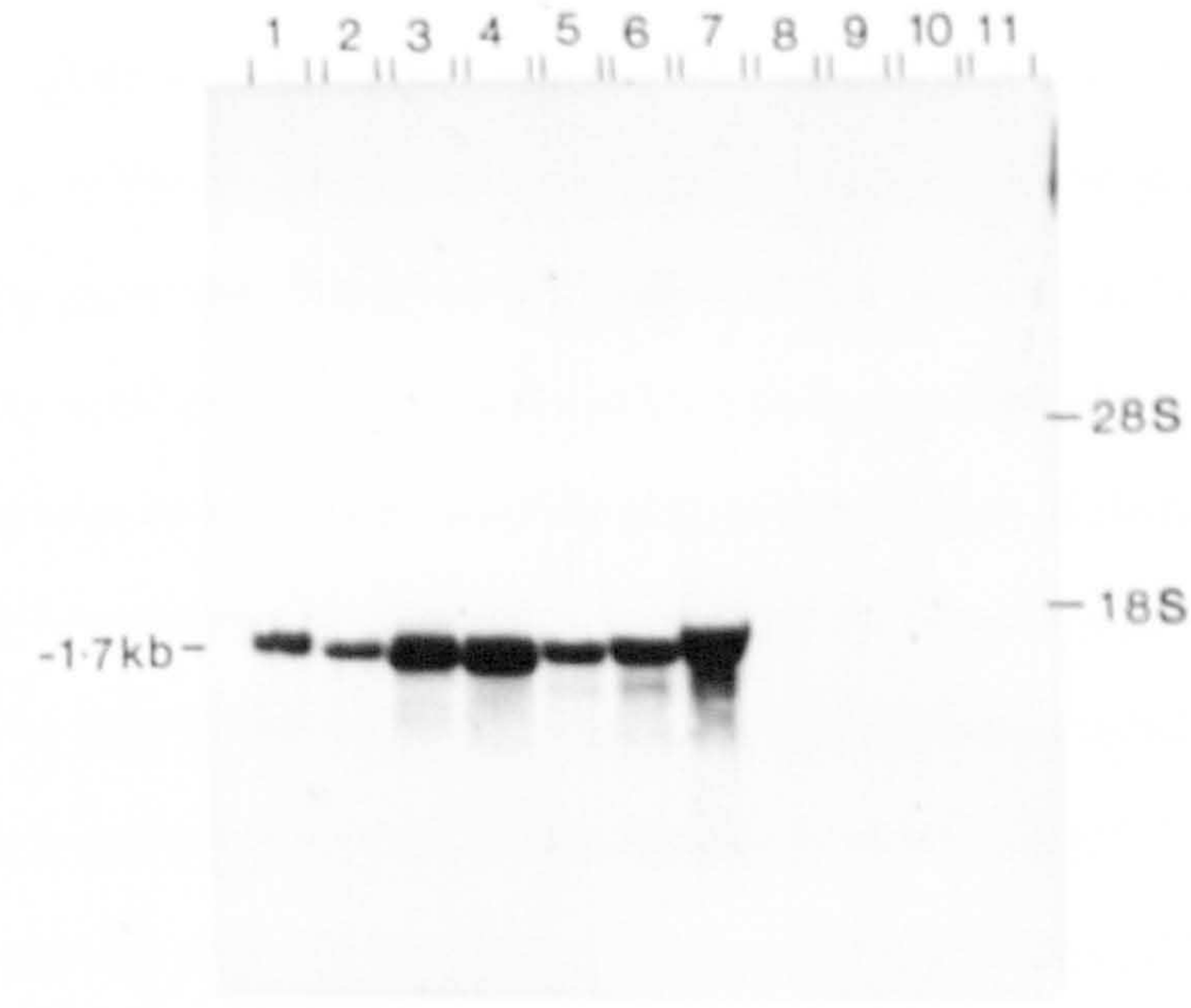


Figure 23: Northern blot of figure 22 reprobed with mouse α-actin cDNA. The hybridization and washing conditions are described in the text. Samples in lanes are indicated in figure 21 and the positions of the ribosomal RNAs are marked.



mRNA at approximately the expected size of 1.7kb with the same specificity unlike the MLC2 cDNA. This suggests that the mouse α-actin cDNA has the same degree of homology to both chicken and bovine α-actin mRNA, which would be expected for this highly conserved protein. The bovine α-actin mRNA appears to be slightly larger than that of the chicken.

Both the chicken MLC2 and mouse α-actin cDNAs were subsequently used on total RNA samples from the bovine cimaterol trial (section 4.2.) to monitor the effects of the β-agonist on their respective mRNA expression in the skeletal muscle.

Because the oligonucleotide I probe would not specifically detect calpain large subunit mRNA in chicken muscle total RNA and bovine muscle total plus poly(A)+ RNA, I attempted to make a calpain large subunit cDNA probe by the polymerase chain reaction.

4.1.2. The Attempted Generation of the Chicken Skeletal Muscle Calpain Large Subunit cDNA by the Polymerase Chain Reaction.

In the Polymerase Chain Reaction a DNA sequence is amplified between two oligonucleotide primers which flank the region, using the thermostable enzyme Taq DNA polymerase in repeated cycles of heat denaturation of DNA, annealing and extension of the PCR oligonucleotides (184). At the time of the attempted production of a calpain large subunit cDNA by PCR there were several reports of the preparation of cDNA by PCR directly from total RNA via first strand cDNA without the need for purification of the first strand cDNA, after its synthesis by reverse transcriptase (181,201).

PCR was to be used to amplify part of the chicken skeletal muscle calpain large subunit mRNA sequence to produce a partial cDNA. The reasons for this approach were:

1) At the time of this work there was only one reported calpain large subunit isoform at the protein and mRNA level in chicken skeletal muscle (12,14), therefore the PCR product would be only for one isoform rather than possible multiple products from a mammalian muscle. More recent reports in the literature have suggested there is a calpain I along with a third isoform enzyme activity in chicken breast muscle (13).

2) Oligonucleotide I, which hybridized to the chicken muscle calpain mRNA sequence, could be used as one of the PCR oligos.

3) The PCR cDNA product was predicted to be an large subunit isoform which is a calpain II-like in action (14) but has a high homology to human calpain I and II at a amino acid level, 70% and 66% respectively (17) (see Table 1). This calpain PCR cDNA could possibly be used to detect both large subunit isoforms from mammalian species on Northern blots. Although the sequence may have the same percentage mismatch as the oligonucleotide I a longer sequence would be more stable in hybridization. Alternatively it could be used to isolate large subunit clones from other species' cDNA libraries.

Oligonucleotide I (39mer) was antisense to the chicken large subunit cDNA sequence at 1437-1475 bp, therefore it became the 3'PCR oligo. Using the protocol as described in section 3.3.4.(i) and shown in Figure 24, first strand cDNA was generated using AMV reverse transcriptase and oligonucleotide I or oligo-d(T) on chicken skeletal muscle total RNA.

The choice of 5'PCR oligo was determined by the position of the oligonucleotide I, the 3'PCR oligo, Figure 18. According to advice given by groups using PCR, a region 1kb upstream of the 3'PCR oligonucleotide was suitable. However this meant the 5'PCR oligo was within Domain II of the cDNA so may have had homology toward other cysteine proteinases (14), see section 2.1.1..

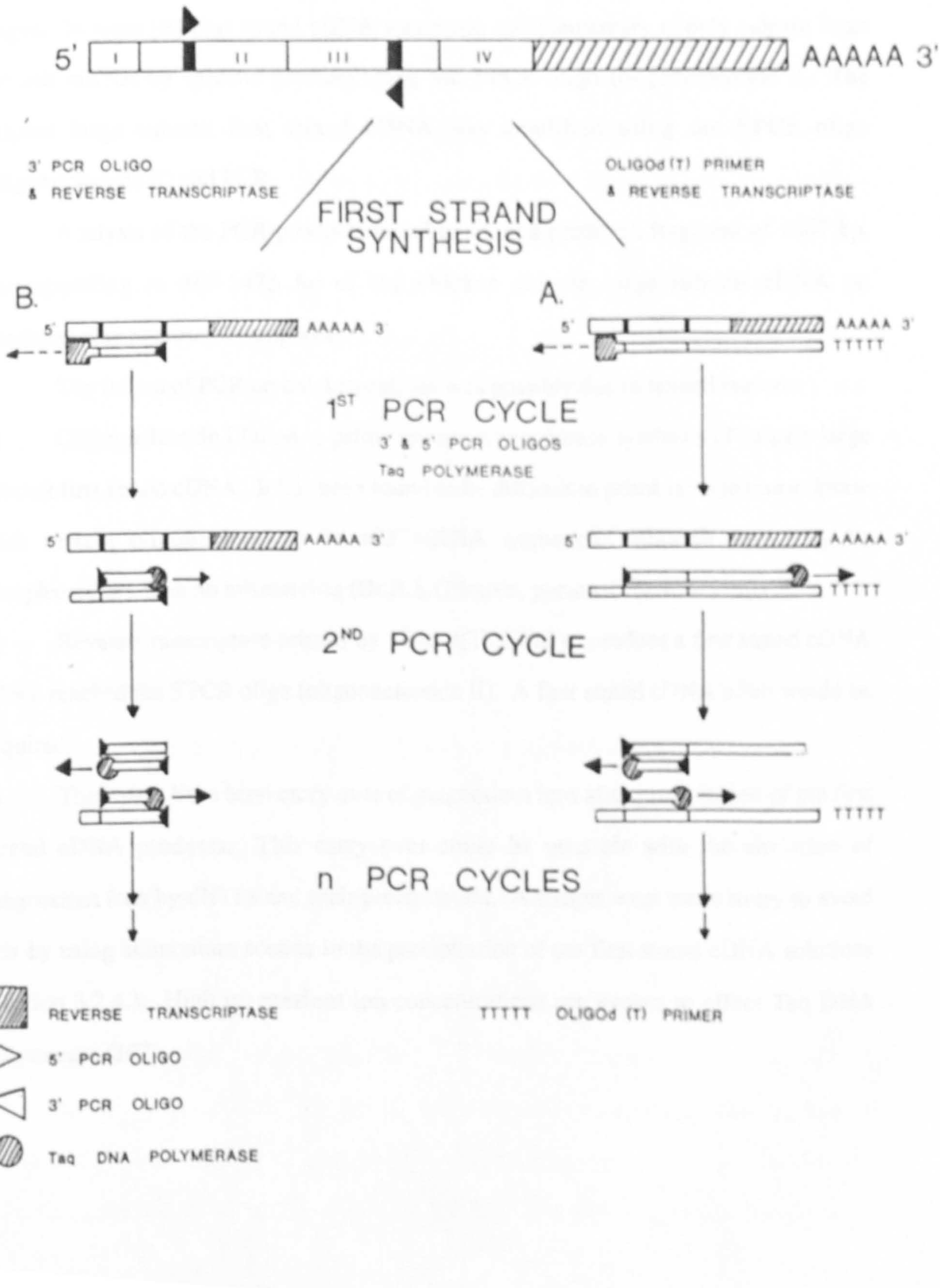
The 5'PCR oligo (oligonucleotide II) selected as 'sense' chicken calpain large subunit sequence and consisted of 30 nucleotides show below;

Oligonucleotide II

chicken calpain large subunit cDNA 469-498 bp.

5' GTG GAC GAC CTG CTG CCC ACC AAG GAC GGG 3'

Figure 24: The amplification of the chicken skeletal muscle calpain large subunit cDNA by the Polymerase Chain Reaction (PCR).



Using the first strand cDNA generated from either oligo-d(T) or oligonucleotide I primers, PCR was carried out (section 3.3.4.(i)). An outline of the two PCR methods used is shown in Figure 24. In the pathway marked [A] first strand cDNA was made to the general pool of skeletal muscle mRNA using oligo-d(T) primers. The calpain large subunit cDNA was then selectively amplified using the calpain-specific 5' and 3' PCR oligos. In route [B] first strand cDNA was made complementary to only calpain large subunit mRNA by specific priming using the 3'PCR oligo (oligonucleotide I). The calpain large subunit first strand cDNA was amplified using the 5'PCR oligo (oligonucleotide II) and PCR.

Analysis of the PCR products failed to show a predicted fragment of 1007 bp, corresponding to 469-1475 bp of the chicken calpain large subunit cDNA on nondenaturing agarose electrophoresis.

The failure of PCR on chicken calpain was possibly due to several reasons:

1) Oligonucleotide I failed to prime reverse transcriptase synthesis of calpain large subunit first strand cDNA. It has been found to be difficult to prime reverse transcriptase with certain oligonucleotides in mid mRNA sequence, although they may be complementary with no mismatches (Dr.R.S.Gilmour, personal communication).

2) Reverse transcriptase primed by oligo-d(T) failed to produce a first strand cDNA which reached the 5'PCR oligo (oligonucleotide II). A first strand cDNA >3kb would be required.

3) There may have been carry over of magnesium ions after precipitation of the first strand cDNA products. This carry over could be possible with the chelation of magnesium ions by dNTPs and their precipitation. Attempts were made to try to avoid this by using ammonium acetate in the precipitation of the first strand cDNA solutions (section 3.2.4.). High magnesium ion concentrations are known to effect Taq DNA polymerase (167).

4.1.3. Synthesis of an Oligonucleotide Probe to Calpain Large Subunit mRNA by Primer Extension.

Although the experiment to generate a cDNA to chicken skeletal muscle calpain by PCR failed, the oligonucleotide II had been selected carefully so that it could be used to produce a new oligonucleotide probe, within the constraints specified by PCR described in the previous section.

The chicken specific calpain large subunit oligonucleotide II (30mer) was chosen to have high homology across species to human calpain I and II large subunit cDNAs so that the probe could be used to detect sequences in other species particularly bovine samples. Rabbit large subunit cDNAs did not cover this area of the sequence (15). The oligonucleotide adopted, oligonucleotide II is shown in Figure 25 along with corresponding regions in the human isoforms.

Table 9 shows the homology of oligonucleotide II to the sequences in the human calpain isoforms.

Using a 12mer;

3' TGG TTC CTG CCC 5'

complementary to the 3'end of oligonucleotide II an antisense probe was made using Klenow fragment and [α-^{32}P]dCTP (3000 Ci/mmol) (Amersham), (section 3.3.3.) (179).

The advantage of generating a probe by this method was it could be made to a higher specific activity than an 5' end labelled probe, such as oligonucleotide I. When making and labelling oligonucleotide II by primer extension the specific activity of the [α-^{32}P]dCTP was reduced by half by adding an equimolar quantity of 'cold' dCTP to 50μCi of [α-^{32}P]dCTP (3000 Ci/mmol) (16.6 pmol). In 5'end labelling up to one mole of ^{32}P or less may be incorporated into one mole of oligonucleotide whilst in primer extension, using diluted isotope, 3 moles of ^{32}P could be incorporated into one mole of antisense oligonucleotide II. By diluting the isotope more antisense oligonucleotide probe was probably made and it still may have been of higher specific activity than the end-labelled probe. With such a probe less total RNA would be required on Northern

Figure 25. The sequence of the chicken muscle calpain large subunit cDNA (14) chosen for Oligonucleotide II and the corresponding region in other calpain large subunit cDNAs (16,17). The numbers indicated in brackets in base pairs.

Oligonucleotide II

from chicken muscle calpain cDNA (469-498)

5' GTG GAC GAC CTG CTG CCC ACC AAG GAC GGG 3'

human calpain I cDNA (490-519)

5' GTG GAT GAC CTG CTG CCC ATC AAG GAC GGG 3'

human calpain II cDNA (460-489)

5' GTG GAT GAC AGG CTG CCC ACC AAG GAC GGG 3'

Table 9. Percentage homology of oligonucleotide II with the cDNAs for the human calpain large subunit isoforms (16,17).

	Homology of oligonucleotide sequence	
	human calpain I	human calpain II
chicken calpain	93%	90%

blots to detect calpain large subunit mRNA, hopefully reducing background caused by nonspecific hybridization.

A comparison of labelling techniques was made:

Reaction I: 30pmol of oligonucleotide I was labelled using 100μCi of [γ-^{32}P] ATP (>5000 Ci/mmol) equivalent to <20pmol using polynucleotide kinase (section 3.3.2.). The products of the reaction were separated by chromatography on Sephadex G-50 (section 3.4.10.), 2μl of each fraction was counted for the activity present and the results plotted on a graph, Figure 26 A (i). Also 5μl of each fraction was run on a denaturing polyacrylamide-urea gel (section 3.4.11.) and autoradiographed, Figure 26 A (ii).

Reaction II: 5.5pmol of oligonucleotide II was copied into the complementary strand using 22pmol of the specific oligo primer and 50μCi of [α-^{32}P]dCTP (3000 Ci/mmol), which was diluted to 1500 Ci/mmol equivalent to 33pmol (section 3.3.3.). The products of the reaction were analysed as described for reaction II above and are shown in Figure 26 B (i) and (ii).

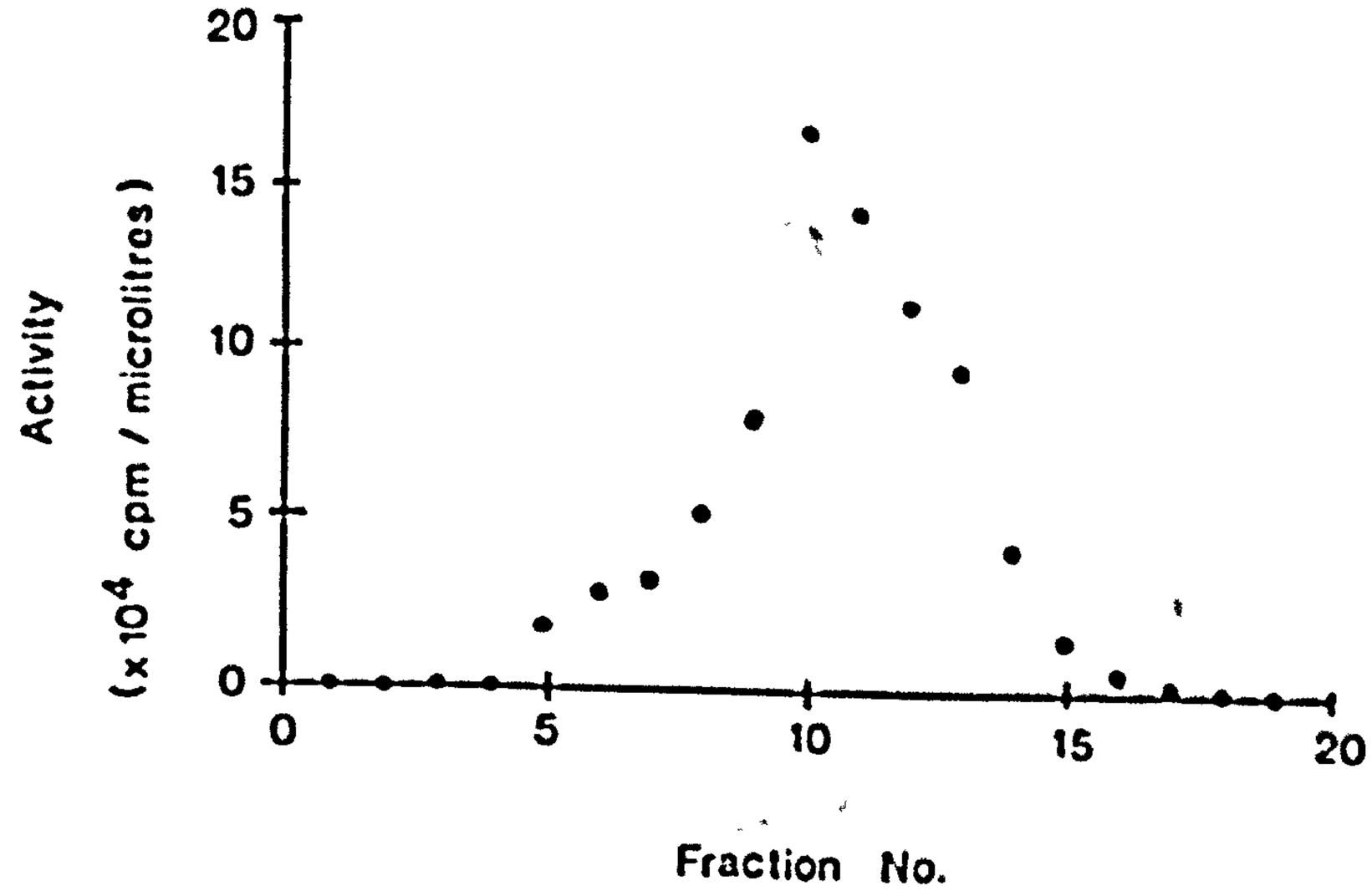
It can be clearly seen that the incorporation of isotope, from the relative intensities of the labelled oligonucleotides to the unincorporated nucleotides, using primer extension method gives probes of high specific activity and ^{32}P incorporation.

The labelled chicken calpain large subunit oligonucleotides were used to probe Northern blots of chicken skeletal muscle total and poly(A)+ RNA. The blots were made from a gel which was electrophoresed with replicate RNA samples on it to give two separate Northern blots which were identical.

The antisense oligonucleotide II primer extension product had to be heat denatured to single stranded DNA before being used for hybridization. To help ensure that the labelled antisense oligonucleotide remained single stranded in the hybridization solution the formamide system was used (section 3.4.12.).

Figure 26: Comparison of oligonucleotide labelling techniques:
A. Reaction I; 5' end labelling of oligonucleotide I.
i) Sephadex G-50 chromatography of labelled products.
ii) Autoradiograph of denaturing polyacrylamide-urea gel electrophoresis of chromatographically separated products.

i)



ii)

origin

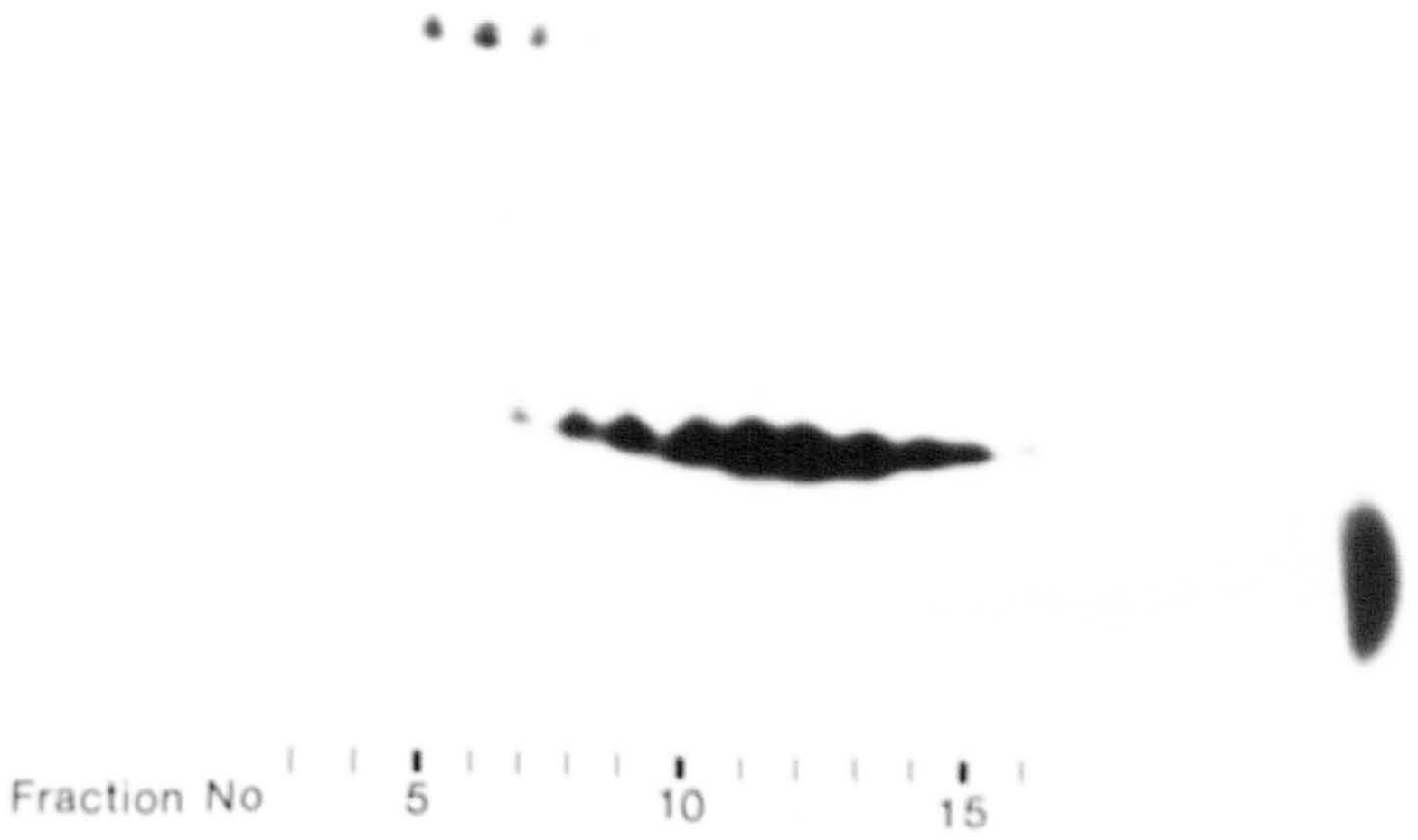


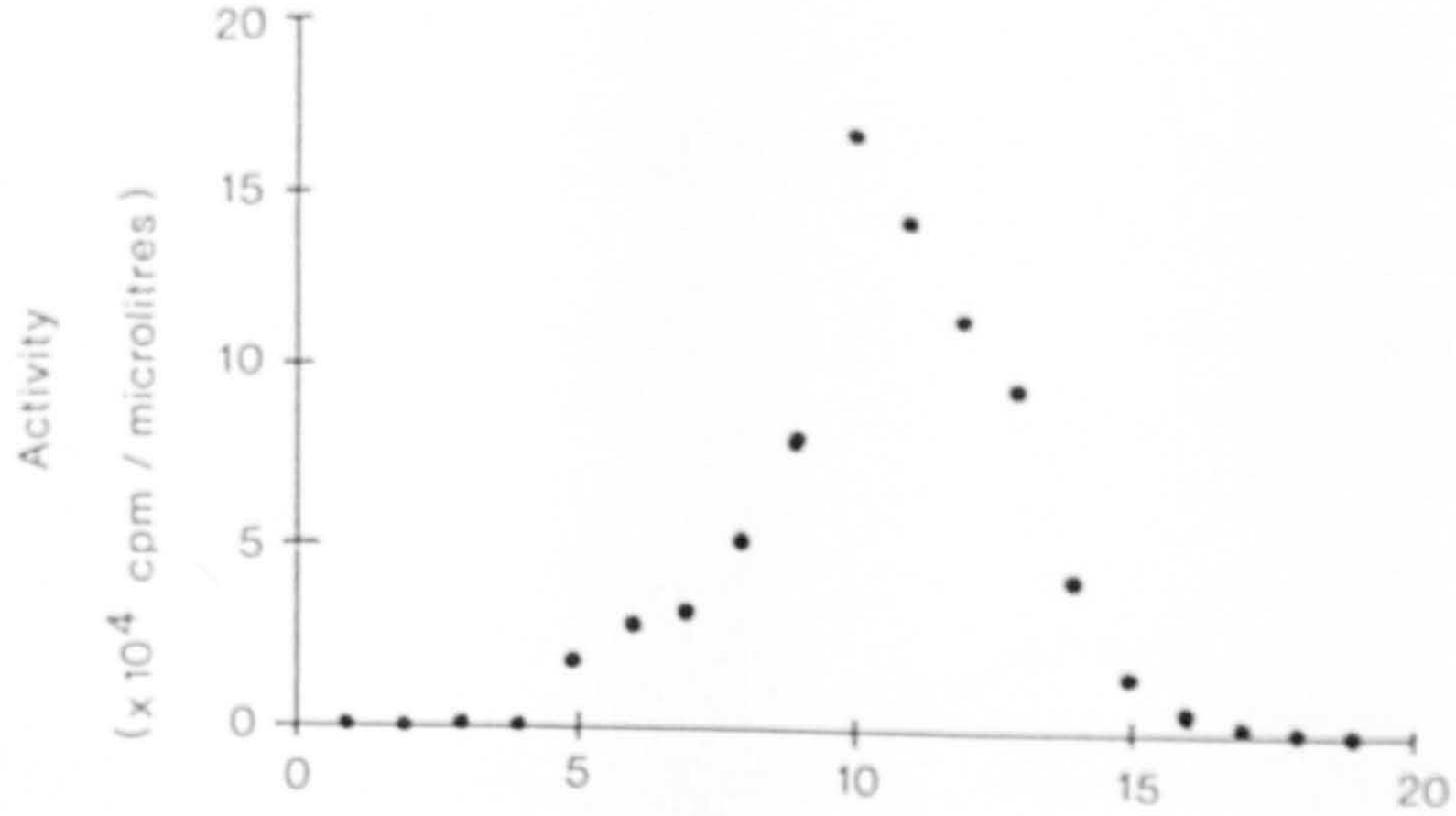
Figure 26: Comparison of oligonucleotide labelling techniques:

A. Reaction I; 5' end labelling of oligonucleotide I.

i) Sephadex G-50 chromatography of labelled products.

ii) Autoradiograph of denaturing polyacrylamide-urea gel electrophoresis of chromatographically separated products.

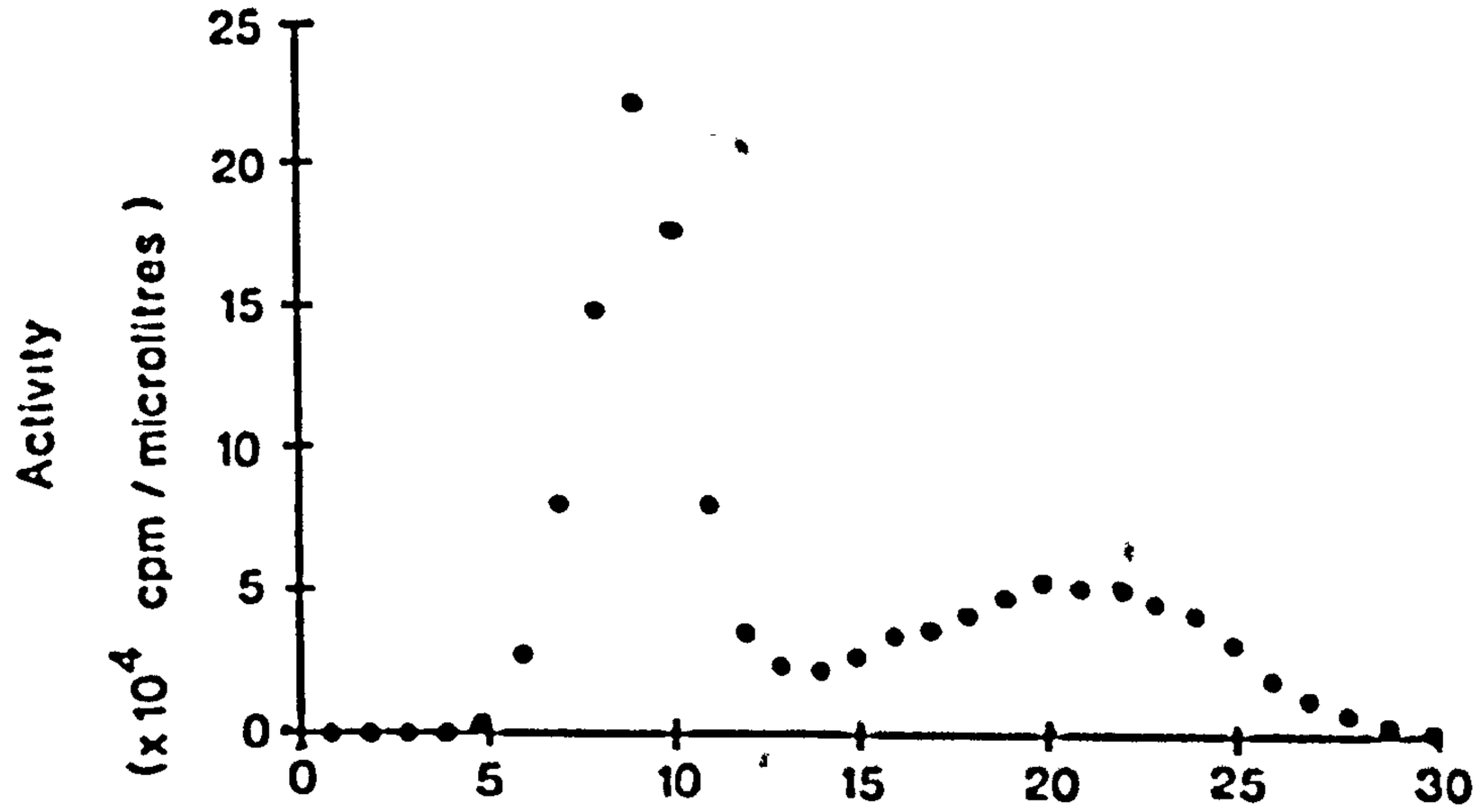
i)



ii)

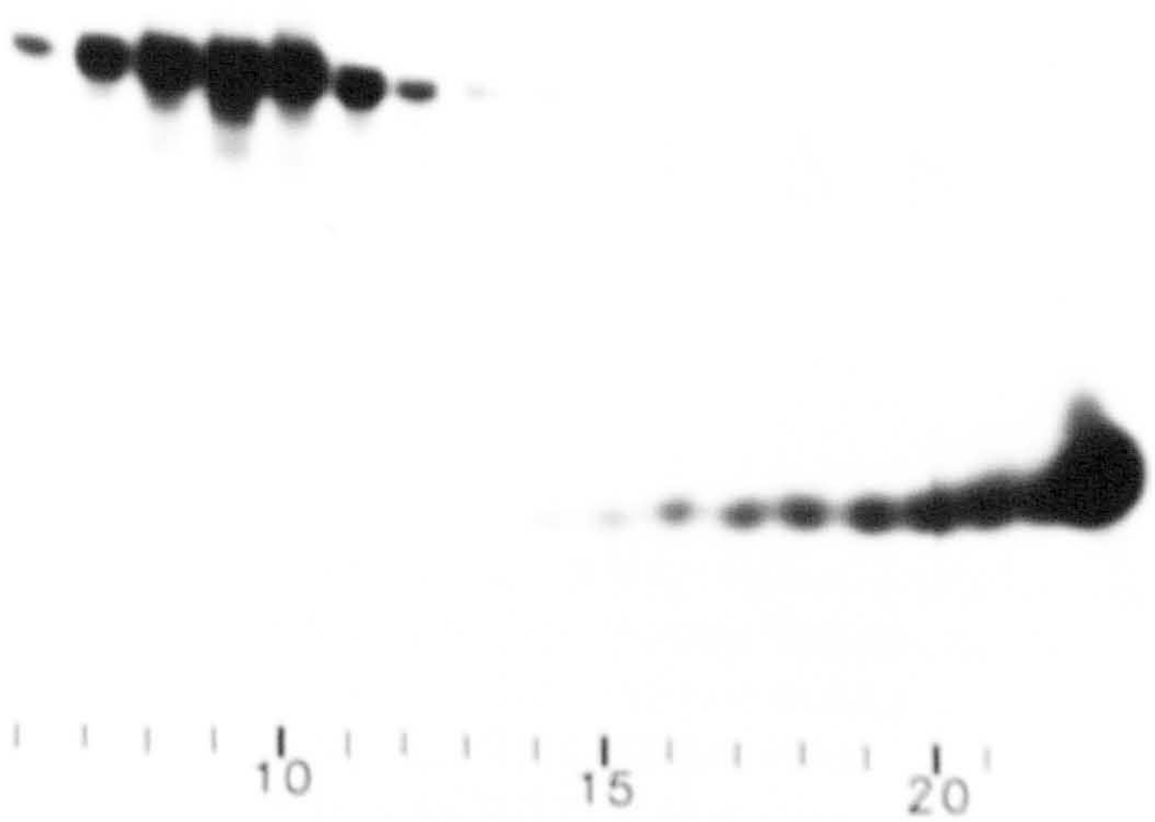
Figure 26: Comparison of oligonucleotide labelling techniques:
B. Reaction II; labelling antisense oligonucleotide II by specific primer extension.
i) Sephadex G-50 chromatography of labelled products.
ii) Autoradiograph of denaturing polyacrylamide-urea gel electrophoresis of chromatographically separated products.

i)



ii)

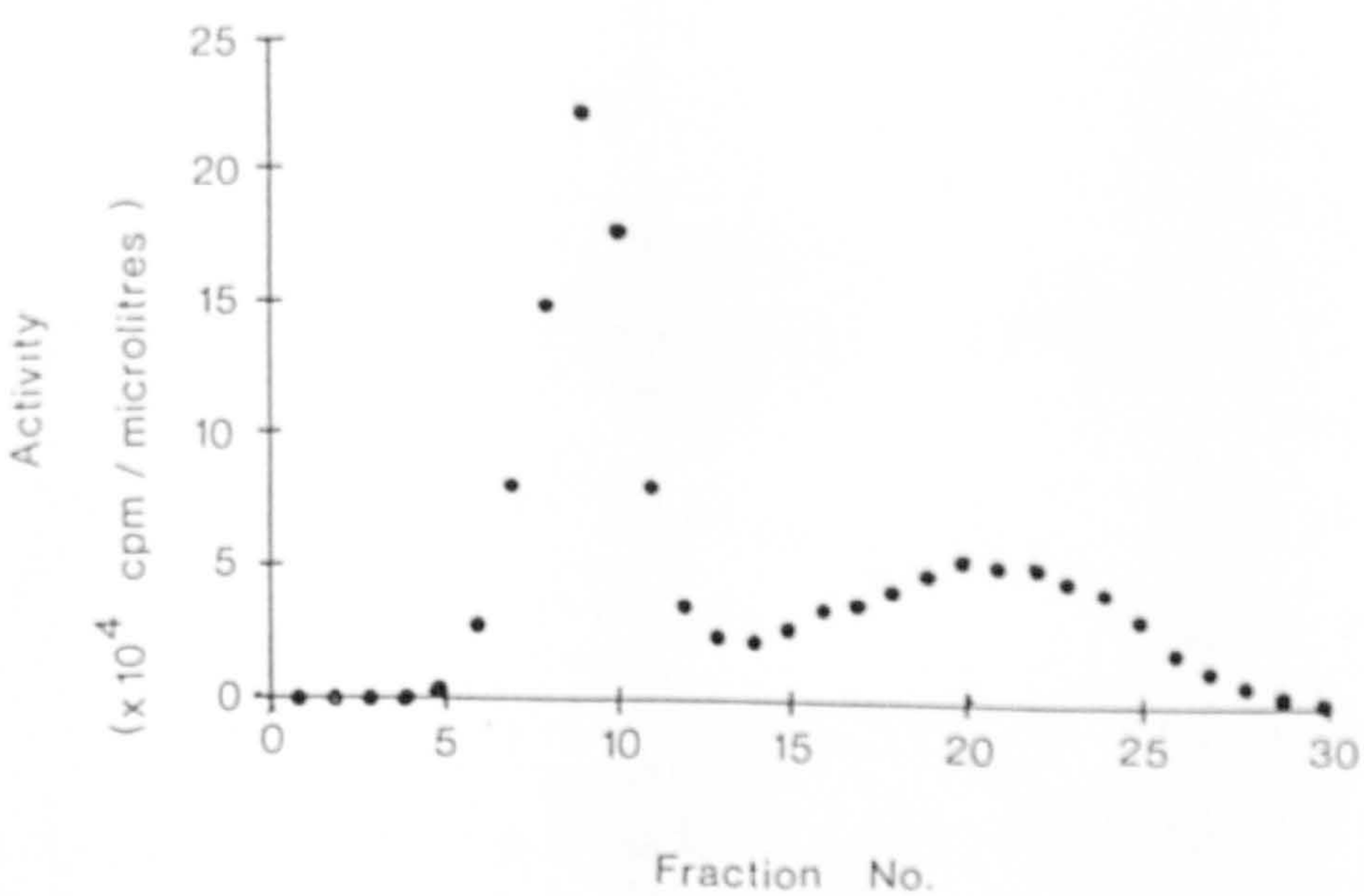
origin _



Fraction No 5 10 15 20

Figure 26: Comparison of oligonucleotide labelling techniques:
B. Reaction II; labelling antisense oligonucleotide II by specific primer extension.
i) Sephadex G-50 chromatography of labelled products.
ii) Autoradiograph of denaturing polyacrylamide-urea gel electrophoresis of chromatographically separated products.

i)



ii)

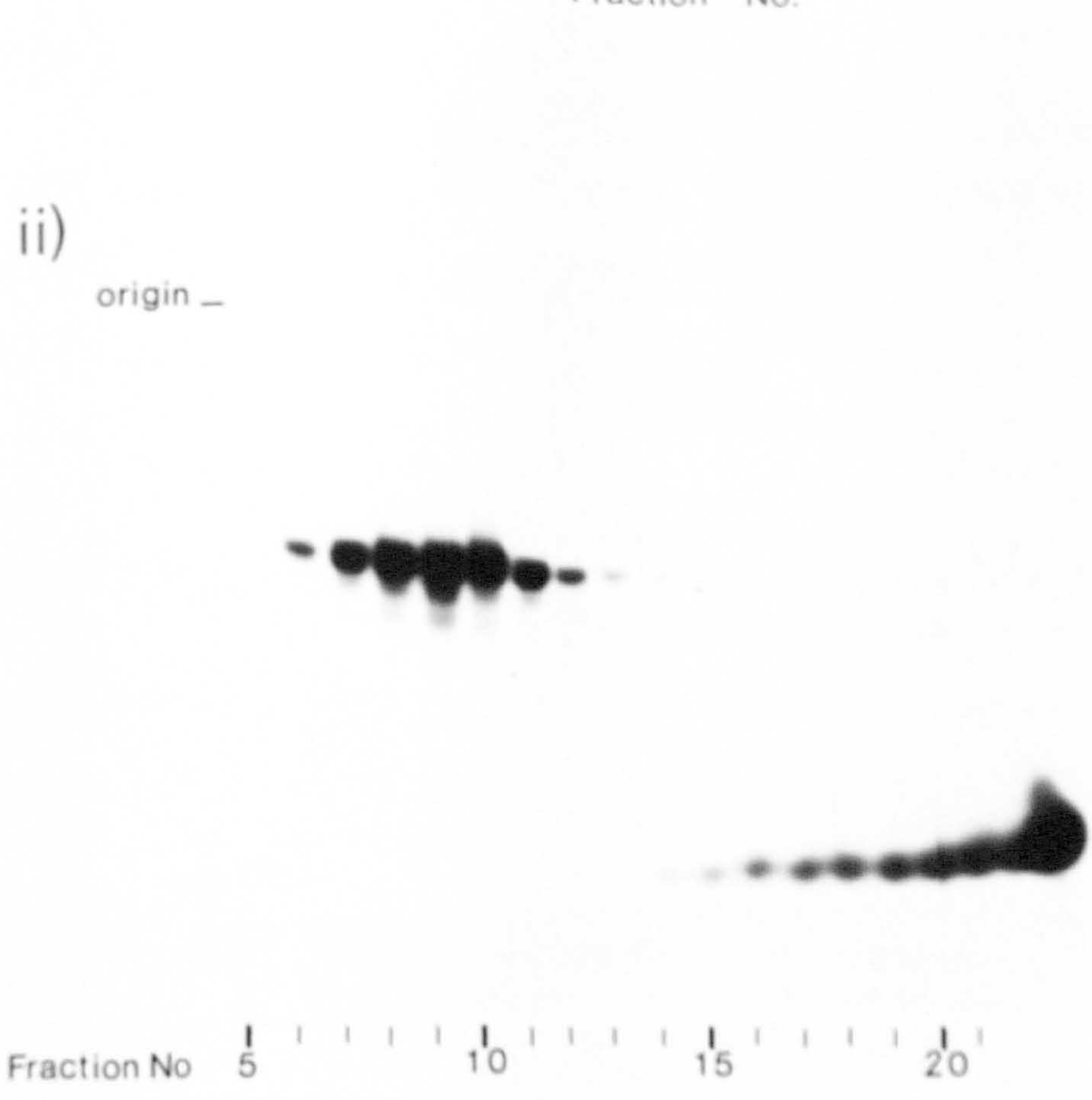
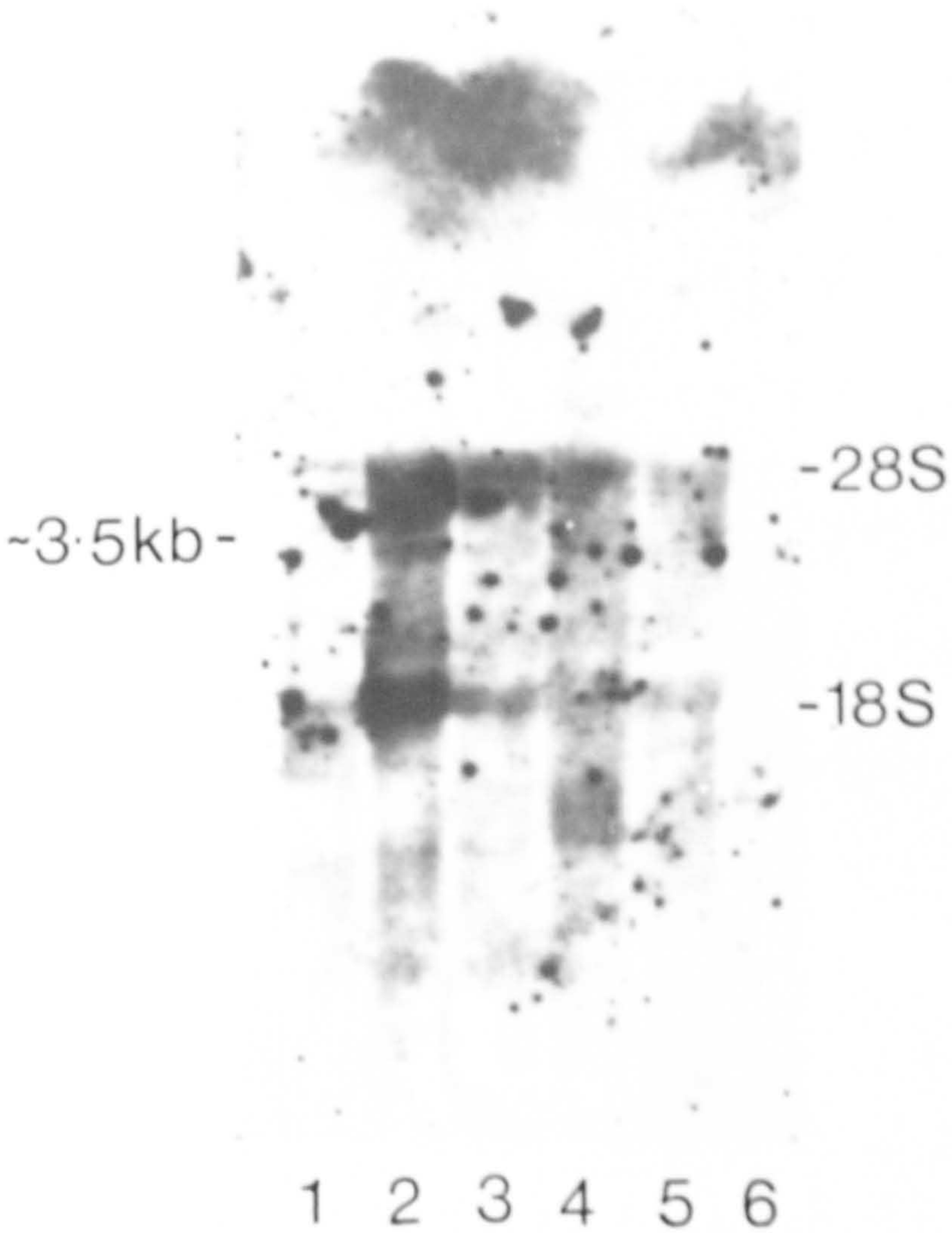
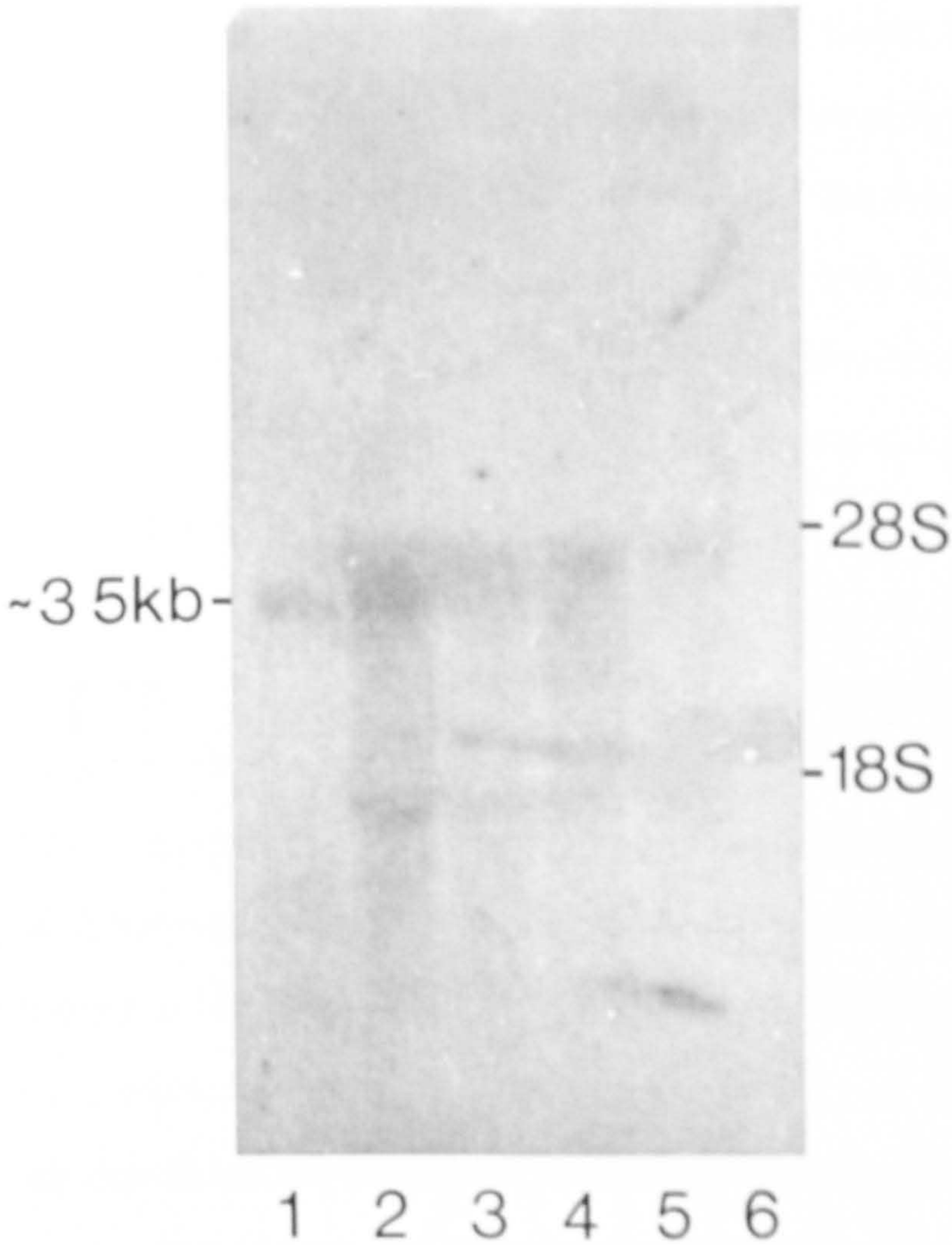


Figure 27: Replicate Northern blot of chicken skeletal muscle total and poly(A)+ RNA each probed with a different calpain large subunit oligonucleotides:
(i). Northern blot probed with oligonucleotide I. The probe was labelled in Reaction I (Figure 26 A.), hybridized at 50°C and washed with 6xSSC at 60°C. The positions of 28S and 18S ribosomal RNAs are indicated.



Lane No.	Sample
1	10µg poly(A)+ RNA, chicken skeletal muscle.
2	50µg total RNA, chicken skeletal muscle.
3	25µg total RNA, chicken skeletal muscle.
4	50µg total RNA, chicken liver.
5	25µg total RNA, chicken liver.
6	1µg HInd III lambda DNA markers.

Figure 27: Replicate Northern blot of chicken skeletal muscle total and poly(A)+ RNA each probed with a different calpain oligonucleotides:
(ii). Northern blot probed with oligonucleotide II. The probe was labelled in Reaction II (Figure 26 B.), hybridized at 42°C in 50% (v/v) formamide then washed up to 5xSSPE plus 0.1% (w/v) SDS at 47°C. The locations of the 28S and 18S ribosomal RNAs are marked.



Lane No.	Sample
1	10µg poly(A)+ RNA, chicken skeletal muscle.
2	50µg total RNA, chicken skeletal muscle.
3	25µg total RNA, chicken skeletal muscle.
4	50µg total RNA, chicken liver.
5	25µg total RNA, chicken liver.
6	1µg Hind III lambda DNA markers.

The autoradiographs of the two different probe hybridizations are shown in Figure 27 (i) and (ii). A faint band is visible at the approximate position of the 3.5kb calpain large subunit mRNA in both autoradiographs as indicated in Figure 27. The hybridization signal appears relatively stronger, particularly in the poly(A)+ RNA tracts, when using the oligonucleotide II probe made by specific primer extension, although the signal is still very weak.

It was apparent that poly(A)+ RNA would have to be used to detect calpain large subunit mRNA in skeletal muscle as it was difficult to distinguish in total RNA. The poly(A)+ RNA used in the blots in Figure 27 was prepared by single passage through oligo-d(T) cellulose so although more was loaded than the Northern in Figure 19, where a strong hybridization signal was seen, it was less enriched with poly(A)+ RNA.

Further development of the primer extension method of labelling may have given better results than seen in these initial experiments. However at this stage the cDNAs of human calpain I and II large subunits were made available so became the probes used in the subsequent experiments.

In summary there were several problems with using oligonucleotide probes to detect the low copy number calpain mRNA:

1) Oligonucleotides had a tendency to bind nonspecifically to noncomplementary nucleic acid sequences (195). Probing total RNA with calpain large subunit oligonucleotides at low stringency gave high background of nonspecific hybridization, particularly to rRNA, in all the methods used.

2) Any significant mismatches in the oligonucleotide probe to the nucleic acid sequence to be detected was sufficient to prevent hybridization. As a result it was difficult to use an oligonucleotide to probe the same isoform sequence across species.

3) There were difficulties in end-labelling the oligonucleotide using polynucleotide kinase to a consistent specific activity as assessed from polyacrylamide/urea gel electrophoresis. This problem has been experienced by other groups within the Faculty and may have been due to the quality of the enzyme. Polynucleotide kinase is known to

be affected by ammonium ions (165) and the water or the sample may have been contaminated.

4) When the specific primer extension method was used to produce a probe of apparent higher specific activity there was a possible problem of the DNA reannealing after being denatured. This could be overcome by separating the two strands on denaturing polyacrylamide gel electrophoresis if the complementary oligonucleotide was used with its 5' oligonucleotide synthesis 'blocking· group' still attached (167,179).

4.1.4. The Use of Human Calpain I and II Large Subunit cDNA in the Detection of the Bovine Calpain Large Subunit Isoforms.

The plasmids containing the human calpain I and II large subunit partial cDNA inserts, p42 and p21-16 (described in appendix A), were provided by Dr Emson of the Institute of Animal Physiology and Genetics Research, Babraham, Cambridge. The cDNAs were isolated by the appropriate restriction endonuclease digest of the plasmids and electroelution (section 3.4.6.and 3.4.7.). They were characterised by restriction endonuclease mapping (appendix A). These cDNAs were then used to try and detect the calpain large subunit mRNAs in bovine skeletal muscle samples as a β-agonist trial in this species was to be analysed.

Bovine skeletal muscle total RNA samples, a range up to 50µg, were electrophoresed on a formaldehyde agarose gel (section 3.2.7.(ii)) where previously glyoxalation of the RNA had been used to denature RNA (section 3.2.7.(i)). By using formaldehyde, electrophoresis could be carried out overnight to ensure good resolution of the RNA within the samples. This method was used for all subsequent denaturing gels.

The resulting Northern blot was probed with human calpain II large subunit cDNA, then reprobed with human calpain I large subunit cDNA (Figure 28 and 29). Both probes were labelled by nick translation (section 3.4.9.(i)). Hybridization was at 55°C for both probes and filters were washed to the same stringency, 6xSSC at 60°C.

Human calpain II large subunit cDNA hybridized to a specific mRNA species at approximately 3.5-3.6kb (Figure 28) the suggested size for calpain II large subunit

Figure 28: Northern blot of bovine skeletal muscle total RNA probed with human calpain II large subunit cDNA. Hybridization and washing conditions are described in the text. The samples loaded are 20, 10, 40 and 50µg total RNA lanes 1 to 4 respectively, along with Hind III lambda DNA markers, 0.5µg, lane 5. The positions of the 28S and 18S ribosomal RNAs are indicated.

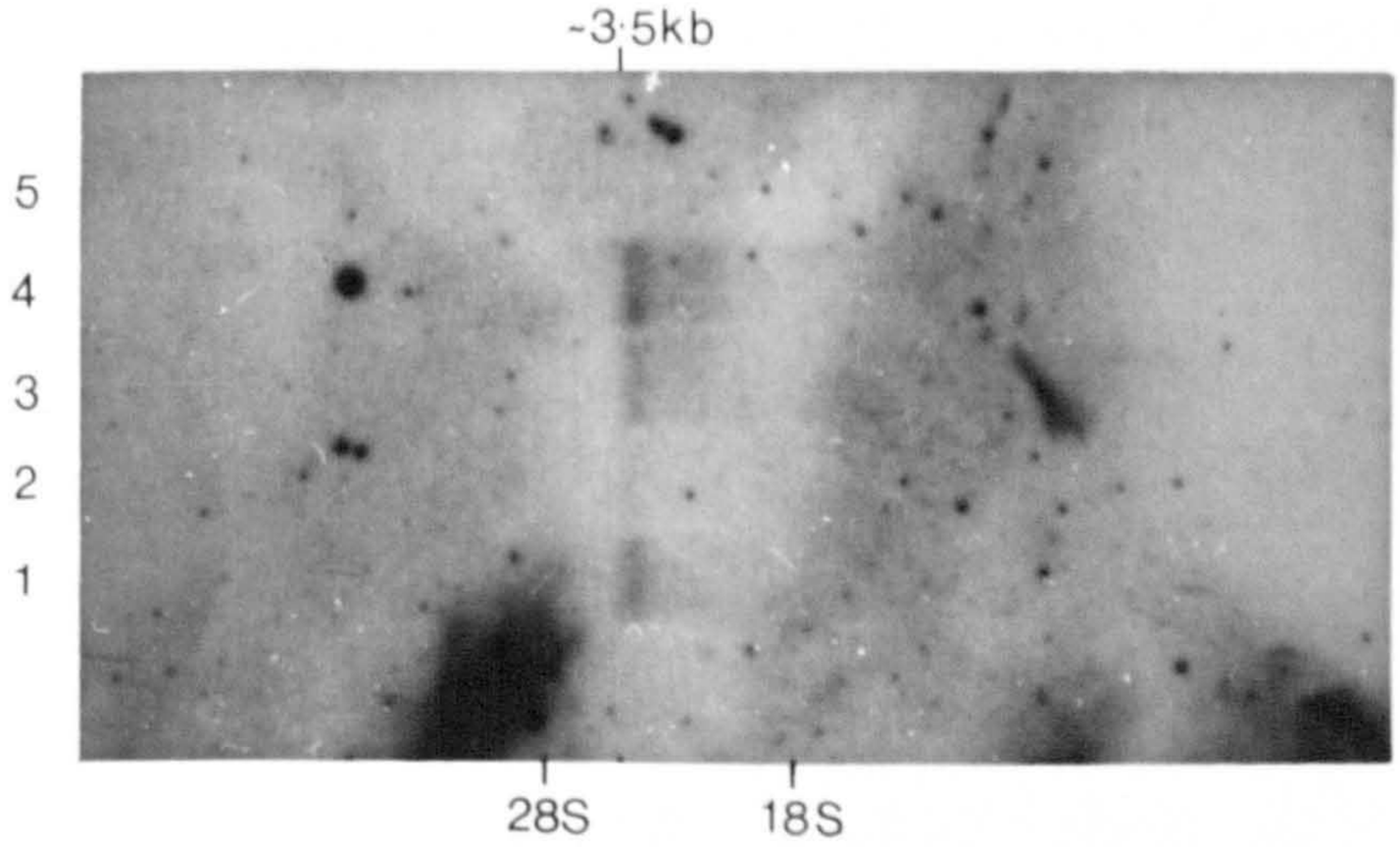
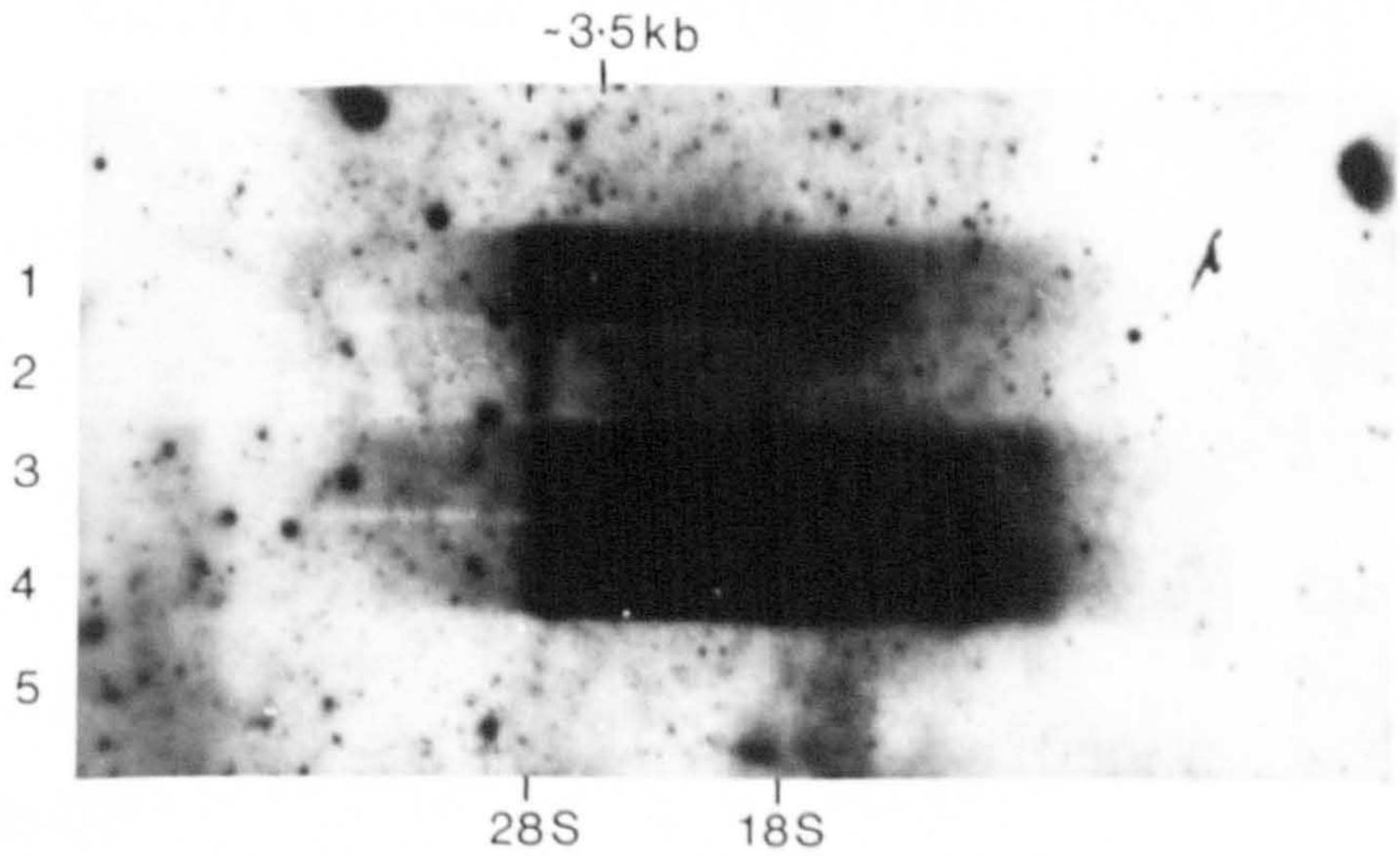


Figure 29: Northern blot in Figure 28 reprobed with the human calpain I large subunit cDNA. The conditions were the same as Figure 28. The locations of the ribosomal RNAs are indication.



mRNAs (15,17). The cross-species hybridization of the human cDNA was not strong, as higher stringency washing, above 2xSSC at 60°C, resulted in the loss of the signal. This was expected as the calpain II large subunit mRNA sequence is not 100% homologous across species. The mismatches lower the T_m from its value for the duplex formation of 100% matching complementary sequences thereby requiring low stringency conditions for hybridization.

The human calpain I large subunit cDNA probe was less specific (Figure 29). The Northern blot was reprobed under the same hybridization and washing conditions as the calpain II. Washing at a higher stringency, 2xSSC at 65°C, failed to remove the background. However faint hybridization bands could be seen near the same position as those when probing with calpain II large subunit cDNA (Figure 29).

To confirm the above observations bovine skeletal muscle poly(A)+ RNA was probed with human cDNAs (Figure 30). Approximately 9µg of poly(A)+ enriched RNA, selected by a single passage through an oligo-d(T) column, was electrophoresed and blotted. Before using the calpain cDNAs on the membrane it had been probed once with a PCR cDNA to bovine calpastatin (section 4.1.5.).

Human calpain II large subunit cDNA hybridized to a specific band at 3.5-3.6kb when the filter was washed to a medium stringency. Using the same hybridization conditions the calpain I large subunit cDNA gave a band at approximately 1.5kb along with nonspecific hybridization, possibly to the small quantity of rRNA which may have remained in the poly(A)+ RNA enriched sample. There was also a very faint band at the expected position of calpain I at approximately 3.5kb (16).

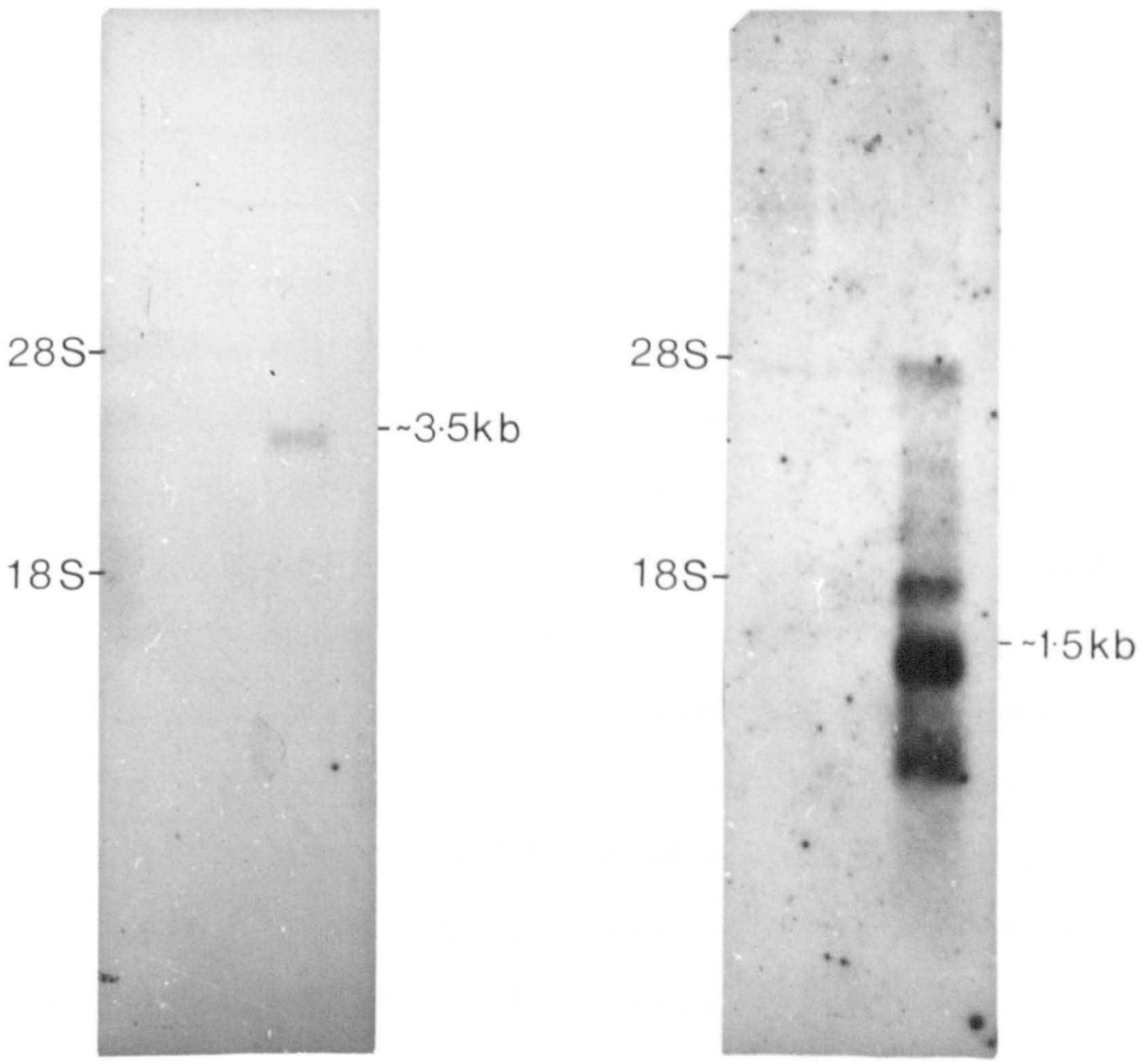
The position of the hybridization band at 1.5kb suggested that calpain I probe may have hybridized to the 30kDa subunit of calpain. The cDNAs for small units of pig and rabbit hybridize to mRNA of 1.6-1.7kb and 1.5kb respectively (18,19), whilst the bovine cDNA is 1.4kb long, but was not reported to be have been analysed by Northern blotting (21).

Figure 30: Northern blot of bovine skeletal muscle poly(A)+ RNA probed with the human calpain large subunit cDNAs.

(i) membrane probed with human calpain II large subunit cDNA.

(ii) membrane reprobed with the human calpain I large subunit cDNA.

Lane 1 and 2 Hind III lambda markers 4µg and 2µg respectively, lane 3, 9µg poly(A)+ RNA, both autoradiographs were hybridized at 55°C and washed in 2xSSC at 60°C. The locations of 28S and 18S ribosomal RNAs are marked.



The nucleotide sequences of domain IV in the rabbit calpain I and II large subunits have 61.9% and 57.3% homology respectively to the small subunit's domain IV' (19) (section 2.1.1.). The clone p42 contains part of the 3' untranslated region of complete calpain I large subunit cDNA including all of domain IV and most of domain III (appendix A). The clone p21-16 contains all of the 3' untranslated region of the complete calpain II large subunit cDNA including part of domain IV (appendix A). As calpain I large subunit cDNA from p42 contains more of domain IV sequence, and has a slightly higher homology to domain IV' (in rabbits), it may hybridize to the small subunit producing the band at 1.5kb. However because of the homology of the primary sequence of Ca^{2+} binding regions in domain IV' to other Ca^{2+} binding proteins, such as troponin and calmodulin, the calpain I cDNA could be hybridizing to their mRNA in the low stringency conditions.

As calpain II cDNA from p21-16 contains all 3' untranslated region this may make it more specific to its corresponding isoform across species. Emori et al (15) who isolated the rabbit calpain large subunit isoforms reported problems when using cDNA nucleotide sequence from amino acid coding regions as probes to distinguish between the mRNA of the two calpain large subunit isoforms. They used 3'noncoding regions as isoform specific probes, which were shown to have no significant sequence homology in rabbit. Although the calpain large subunits used in this thesis were across species this observation may account for the failure of the calpain I large subunit cDNA.

The specificity of the human calpain II large cDNA was assumed to be to its analogous isoform in bovine total RNA for the reasons outlined above. However the cDNA may have been hybridizing to both calpain large subunit isoforms as the human calpain I and II mRNA are suggested to be approximately the same size (3.5kb) (16,17). There also may have been hybridization to the skeletal muscle specific calpain-like mRNA, p94 (28,29). However this has low amino acid sequence homology to calpain I and II large subunits.

The cDNA for human calpain II large subunit was far more specific and sensitive than the oligonucleotide probes even though it was used at low stringency. The human

calpain I large subunit cDNA was nonspecific and behaved like the oligonucleotides when used on total RNA giving high nonspecific background. Although the cDNAs were to be used across species for analysis of the skeletal muscle calpain large subunit mRNAs in a bovine β-agonist trial (section 4.2.5.) they promised to be more sensitive than the oligonucleotide probes, particularly the human calpain II large subunit cDNA.

4.1.5. Generation of a Bovine Calpastatin cDNA (a Partial Sequence) by the Polymerase Chain Reaction.

As calpastatin cDNA sequences had been published in the literature (52-54) but had not been made available to our group, there were several possible strategies which could have been employed to produce a calpastatin mRNA specific hybridization probe:

1) The use of oligonucleotides to the calpastatin cDNA sequence.
2) Generation of a cDNA library then isolation of clones containing the cDNA for calpastatin.
3) The use of PCR to generate a calpastatin cDNA.

Option (1) would be difficult, given the problems described above in attempting to use oligonucleotide probes for calpain across species (section 4.1.1 and 4.1.3.). As the calpastatin cDNA was required primarily as a hybridization probe, option (3) was considered the best way to proceed. Although option (2) would have generated a specific cDNA it was not likely to be as quick as option (3).

The calpastatin cDNA required was to the bovine mRNA, as samples from a bovine β-agonist trial were available. The cDNA sequences in the literature were to rabbit (52), pig (53) and human (54). It was reasoned that because of the homology between the cDNAs (68-78%) if PCR oligonucleotides were selected from conserved sequences across species they might be used to amplify the corresponding sequence from other species by PCR. It had been reported in the literature that oligonucleotides with up to 20% base pair mismatches to their target sequence could be used successfully (182).

Generation of a bovine calpastatin cDNA by PCR using first strand cDNA generated from mRNA was attempted (section 3.3.4.(ii)). The 3'PCR oligo was selected at the 3'end of the coding sequence and the 5'PCR oligo 500bp upstream. The position of the PCR oligonucleotide primers was dictated by the ability of reverse transcriptase to generate first strand cDNA of sufficient length which would reach the 5'PCR oligo, and the selection of published cDNA sequences which were homologous in the amino acid coding region. Since the PCR sequence was to be 500bp and the calpastatin cDNAs reported in the literature had a long 3' noncoding region of approximately 1.5kb (52,53) reverse transcriptase would have had to generate a first strand of at least 2kb. A longer PCR sequence with its 5'PCR oligo toward the 5' end of the mRNA would consequently result in reduced proportion of the first strand cDNAs reaching this region.

The PCR oligonucleotides (17mers) were sense (5'PCR oligo) and antisense (3'PCR oligo) to the pig calpastatin cDNA and had been selected to have high homology to the corresponding sequences in rabbit and human as shown in Figure 31.

The position of the PCR oligonucleotides in the pig cDNA are shown in Figure 32, along with the relative sizes of the other calpastatin cDNAs, and the positions of the predicted PCR amplification products that would be generated using the PCR oligos. The selected PCR oligos spanned domain 4 in all three cDNAs so that the resulting PCR product would contain the sequence for a complete inhibitory domain, one of the four.

Figure 31. Selection of 5' and 3' PCR oligonucleotides for the generation of a bovine calpastatin cDNA. Oligonucleotides were taken from the pig calpastatin cDNA (53) and are shown in comparison to the corresponding sequences of the rabbit (52) and human (54) calpastatin with the mismatching nucleotides underlined.

5'PCR oligo

pig calpastatin cDNA 1609-1625bp

5' GCT GCC GTC TCT GAA GT 3'

rabbit calpastatin cDNA 1621-1637bp

5' GCT GCC GTC TCΔ GAA GT 3'

human calpastatin cDNA 1504-1520bp

5' GCT GCC ΔTC TCT GAA GT 3'

3'PCR oligo

(the complementary sequence to that shown below was used)

pig calpastatin cDNA 2071-2087bp

5' AAA GCA AAG GAT TCC AC 3'

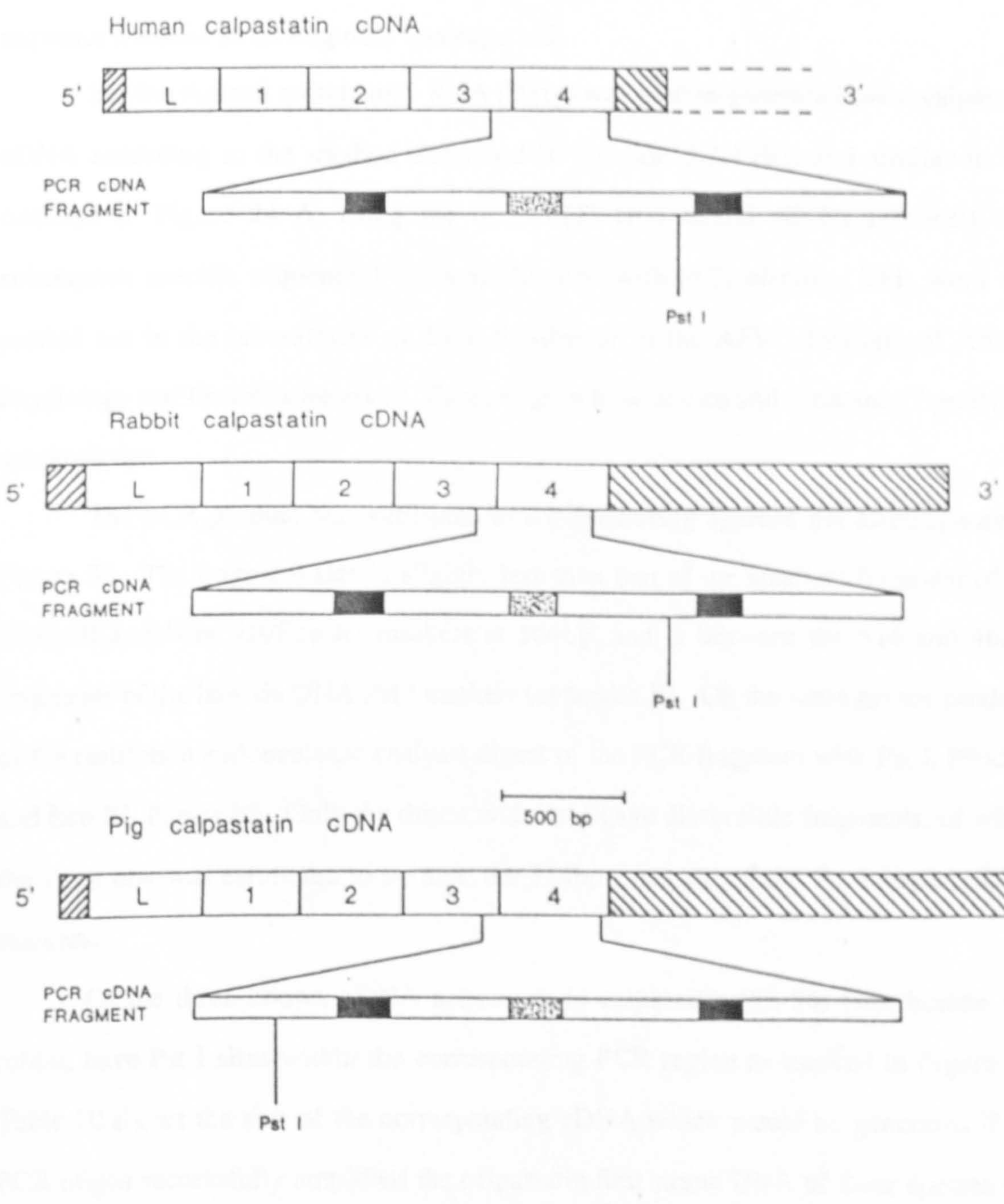
rabbit calpastatin cDNA 2086-2102bp

5' AAA GCA AAG GAT TCΔ GC 3'

human calpastatin cDNA 1966-1982bp

5' AAA GCG AAG GAT TCΔ GC 3'

Figure 32: The PCR cDNA that would be generated from pig rabbit and human calpastatin cDNAs using the calpastatin PCR oligos. The subdomain conserved regions are indicated in the PCR cDNA for each species as well as the Pst I recognition site position.



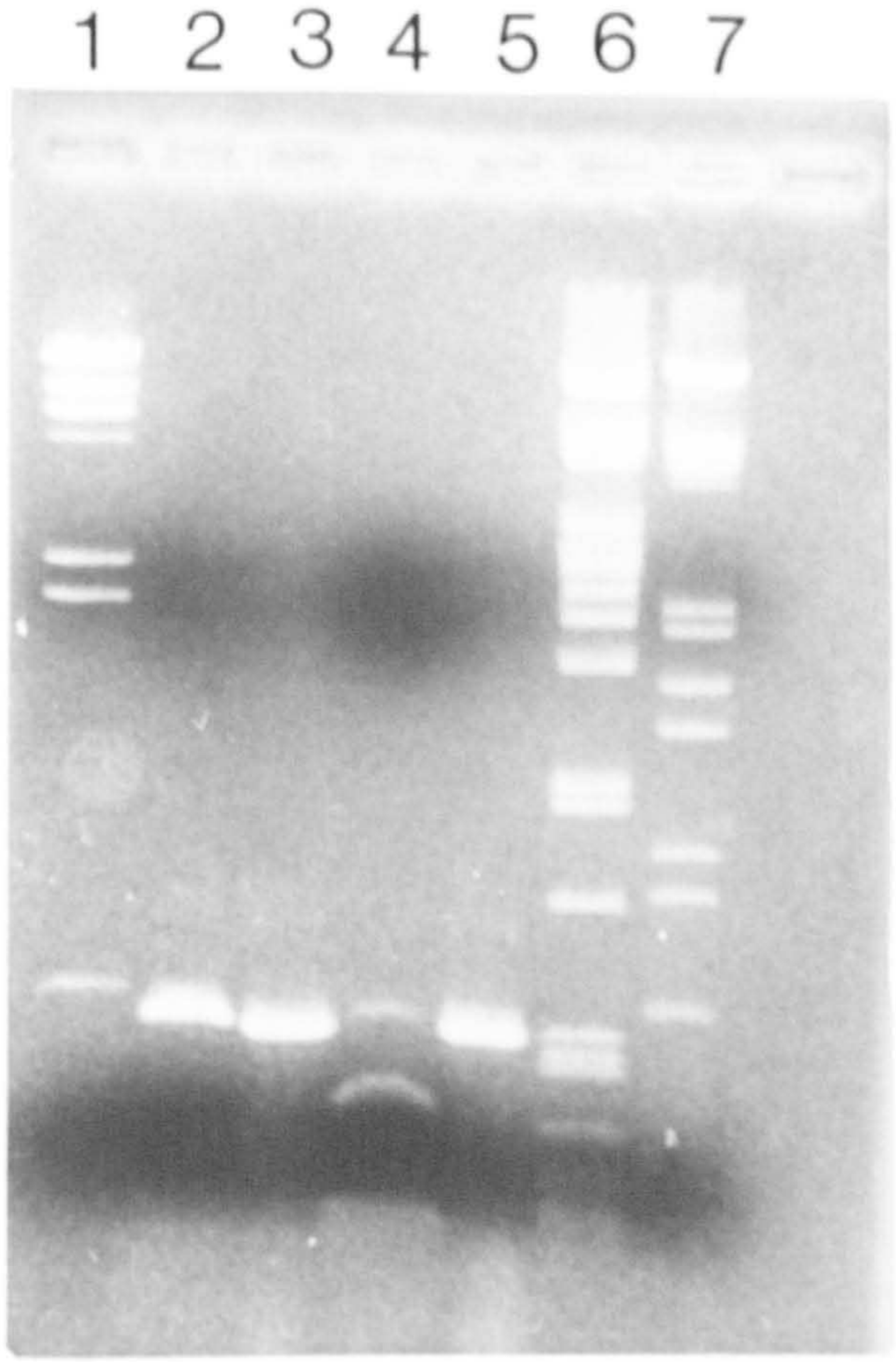
To each of the PCR oligos a restriction endonuclease recognition site sequence was added, a Hind III to the 5'PCR oligo and an Eco RI to the 3'PCR oligo. Using the PCR oligonucleotides on pig first strand cDNA a PCR fragment of 479bp would be expected. Applying them across species, on the rabbit and human, would generate sequences of 482 and 479bp respectively. Therefore on an unknown calpastatin mRNA sequence a similar sized fragment was expected.

Bovine skeletal muscle total RNA (20µg) was used to generate bovine calpastatin cDNA according to the method described in (section 3.3.4.(ii)) and similar to that outlined in Figure 24 A, using the oligo-d(T) first strand cDNA synthesis then subsequent specific sequence PCR amplification with PCR oligos. This work was carried out in the laboratories of Dr R.S.Gilmour in the AFRC Institute of Animal Physiology and Genetics Research, Cambridge, whose advice and assistance I gratefully acknowledge.

The PCR product was subjected to nondenaturing agarose gel electrophoresis, Figure 33. The fragment size is slightly less than that of the smallest fragment of the Hind III and Hind III/Eco RI markers at 564bp, and is between the 514 and 468bp fragments of the lambda DNA Pst I markers (appendix B). On the same gel are products of the restriction endonuclease analysis digest of the PCR fragment with Pst I, Hind III and Eco RI, Figure 33. Only the digest with Pst I gave discernible fragments, of which the large one was estimated to be near the 334bp fragment of the Pst I lambda DNA markers.

Of the three known cDNA sequences to calpastatin (52-54) two, human and rabbit, have Pst I sites within the corresponding PCR region as marked in Figure 32. Table 10 shows the size of the corresponding cDNA which would be generated if the PCR oligos successfully amplified the calpastatin first strand DNA of these species and the size of the Pst I fragment from a digest of the predicted PCR cDNA. This restriction endonuclease data partially confirmed that the PCR cDNA was that of bovine calpastatin. To verify this the cDNA was sequenced after being cloned into the plasmid vector pGEM3 (Promega).

Figure 33: Nondenaturing 1% agarose gel electrophoresis of the PCR fragment generated from bovine skeletal muscle total RNA along with the products of its restriction endonuclease analysis. The markers are Hind III Eco RI/Hind III and Pst I fragments of lambda DNA (appendix B).



1 Hind III lambda DNA markers
2 Eco RI digest of PCR fragment
3 Hind III digest of PCR fragment
4 Pst I digest of PCR fragment
5 PCR fragment
6 Pst I lambda DNA markers
7 Eco RI/Hind III lambda DNA markers

Table 10. The size of the PCR fragment which would be generated from the published calpastatin cDNAs, their position in the full length cDNA and the location of the Pst I site.

Species	Size of PCR fragment*	Position in cDNA	Size of PCR Pst I fragments*	Position of Pst I site in cDNA
pig	479bp	1608-2087bp	425+54bp	1662bp
human	479bp	1504-1982bp	327+152bp	1830bp
rabbit	482bp	1621-2102bp	327+155bp	1947bp

* The values do not include the restriction endonuclease sequences added to the 5'end of the PCR oligonucleotides which would be amplified with the cDNA sequence.

Taq DNA polymerase has no proof reading capability 3' to 5', and this generates a base substitution and one base frameshift error at a rate 1/9000 and 1/41000 respectively during a single round of DNA replication (202). Therefore the PCR reaction was repeated but with replicates. From three replicates the cDNA was treated with Hind III and Eco RI to produce 'sticky ends' which were ligated into the multiple cloning site of the in vitro transcription vector pGEM3 (Promega), between the SP6 and T7 RNA polymerase promoters (section 3.4.1.). The map of the plasmid containing the cDNA insert is shown in Figure 34. The three separate vectors were then transfected into the E.coli strain JM 109 (section 3.4.2.). One of the ligation reactions failed resulting in two clones pG3p13 and pG3p21 containing the PCR products of two reactions. The complete insert was successfully removed from the clones by Hind III and Eco RI restriction endonuclease digest.

The 3' and 5' PCR oligos and the oligonucleotides complementary to the SP6 and T7 promoters were used as primers in dideoxy chain termination sequencing (section 3.4.8.) to give the complete sequence, including the PCR oligo primer regions, of the bovine calpastatin PCR cDNA. Sequencing was carried out on both pG3p21 and pG3p13 plasmids. There were incompatible bases within the novel cDNA sequence possibly produced by PCR errors. However they were at 'wobble positions' and do not effect the deduced primary sequence as shown in Figure 35.

The novel bovine sequence between the PCR oligo primers consists of 451bp equivalent to 150 amino acids. The corresponding regions from pig, rabbit and human cDNAs were aligned to give the best homology to the bovine calpastatin PCR cDNA novel sequence. All the corresponding calpastatin cDNA regions had high homology to the bovine PCR cDNA, particularly the pig and human sequences (Table 11).

Figure 34: The plasmid map of pG3p21 and pG3p13. Numbers in the diagram are in base pairs.

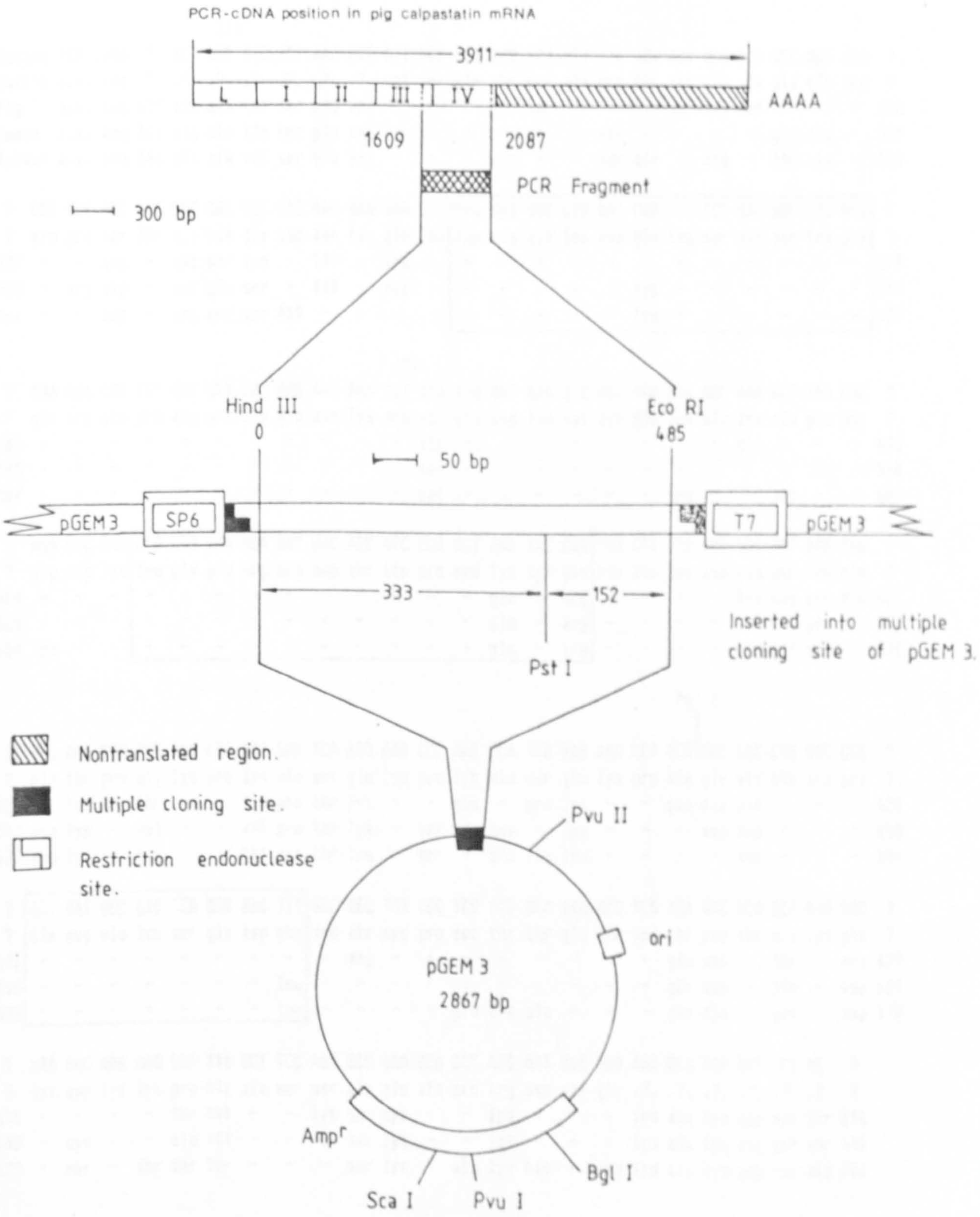


Figure 35: The nucleotide sequence of the PCR generated bovine calpastatin partial cDNA. The deduced primary sequence is aligned with the corresponding primary regions of pig, human and rabbit cDNA (52-54). The dashes indicate identical residues across the species, asterisks indicate gaps introduced to achieve maximum homology. The sequence shown is that of the pG3p21 cDNA insert with nucleotide mismatches to pG3p13 insert shown above. The conserved alpha-helical and inhibitory central consensus sequences are highlighted and are the regions predicted by Maki et al (59,61).

```
                                                (T)
Bovine PCR cDNA  ?  GCT GCC GTC TCT GAA GTG GTT TCC CAA ACC CCA GCT CCA ACC ACC CAG GCA GCC GGT CCA  ?
Bovine a.a. seq  ?  .?. .?. .?. .?. .?. .?. val ser gln thr pro ala pro thr thr gln ala ala gly pro  ?
Pig    a.a. seq 537 ala ala val ser glu val  -   -   -   -  ser  -   -   -   -  his ser  -   -   - 556
Human  a.a. seq 502 ala ala ile ser glu val  -   -   -   -   -   -  ser  -   -   -   -  gly ala  - 521
Rabbit a.a. seq 541 ala ala val ser glu val  -   -   -  ser  -   -  ser ile  -  arg  -  thr ala  - 560

 ?  CCC CCC AGC ACT GCG CAC GGT GAC AAC AAA GAA CTT GAC GAT GCC CTG GAT CAA CTT TCT GAC AGT CTC GGG  ?
 ?  pro pro ser thr ala his gly asp asn lys glu leu asp asp ala leu asp gln leu ser asp ser leu gly  ?
557  -   -  asp  -  val ser asp  -  ttt  -  lys  -   -   -   -   -   -   -   -   -   -   -   -   - 579
522  -  arg asp  -  ser gln ser  -  ttt  -  asp  -   -   -   -   -   -  lys  -   -   -   -   -   - 544
561  -   -  asp  -  arg pro ser ttt  -   -   -   -   -   -   -   -   -  lys  -   -   -   -   -   - 583

                                                (C)
 ?  CAA AGA CAG CCT GAT CCA GAT GAG AAT AAA CCT GTA GAG GAT AAA GTC AAG GAA AAA GCC AAA GCT GAA CAC  ?
 ?  gln arg gln pro asp pro asp glu asn lys pro val glu asp lys val lys glu lys ala lys ala glu his  ?
580  -   -   -   -   -   -   -   -   -   -   -  ile  -   -   -   -   -   -   -   -  glu  -   -   - 603
545  -   -   -   -   -   -   -   -   -   -   -  met  -   -   -   -   -   -   -   -   -   -   -   - 568
584  -   -   -   -   -   -   -   -   -   -   -  met  -   -   -   -   -   -  arg  -   -  lys  -   - 607

 ?  AGA GAC AAG CTG GGA GAA AGA GAT GAC ACC ATC CCA CCT AAA TAC CAA CAT CTT TTG GAT GAC AAC AAG GAG  ?
 ?  arg asp lys leu gly glu arg asp asp thr ile pro pro lys tyr gln his leu leu asp asp asn lys glu  ?
604  -   -   -   -   -   -   -   -   -   -   -   -   -  glu  -  arg  -   -   -   -  lys asp glu glu 627
569  -   -   -   -   -   -   -   -   -   -   -   -   -  glu  -  arg  -   -   -   -   -   -  gly  - 592
608 lys  -   -   -   -   -   -   -   -   -   -   -   -  glu  -  arg  -   -   -   -  gln gly glu  - 631

                                                                                  Pst I
 ?  GGC ACA CCC GGG AAG CCA AAG GCA TCA GAG AAG CCC AAG GCA TCA GAG AAA CCT GCA|GGT GCC CAG GAC CCC  ?
 ?  gly thr pro gly lys pro lys ala ser glu lys pro lys ala ser glu lys pro ala gly gly gln asp pro  ?
628  -  lys ser thr  -   -  ttt pro thr lys  -   -  glu  -  pro lys  -   -  glu ala ala  -   -   - 650
593 asp lys  -  val  -   -  ttt pro thr lys  -  ser glu asp  -  lys  -   -   -  asp asp  -   -   - 615
632 asp lys  -  glu  -   -  ttt pro thr lys  -  ser  -  glu ile lys  -   -   -   -  asp  -   -   - 654

 ?  ATT GAT GCC CTC TCA GGG GAC TTT GAC AGC TGT CCC TCG ACT ACA GAA ACC TCG ACA GAC ACA CCA AAG GAC  ?
 ?  ile asp ala leu ser gly asp phe asp ser cys pro ser thr thr glu thr ser thr asp thr pro lys glu  ?
651  -   -   -   -   -   -   -   -   -  arg  -   -   -   -   -   -   -   -  glu asn  -  thr  - asp 674
616  -   -   -   -   -   -   -  leu  -   -   -   -   -   -   -   -   -   -  gln asn  -  ala  - asp 639
655  -   -   -   -   -   -   -  leu  -   -   -   -  pro ala ala  -   -   -  gln ala  -  glu  - asp 678

 ?  AAA GAC AAG AAG CCT TTT GCT TCC AGT GCC GAA GCA CCT AGG AAT GGC GGG AAA GCA AAG GAT TCC AC   ?
 ?  lys asp lys lys pro ttt ala ser ser ala glu ala pro arg asn gly gly .?. .?. .?. .?. .?. .?.  ?
675  -   -   -   -  thr ttt  -   -  lys ser lys  -   -  lys  -   -   -  lys ala lys asp ser thr 696
640  -  cys  -   -  ala ttt  -   -   -  ser lys  -   -  lys  -   -   -  lys ala lys asp ser thr 661
679  -  ser  -  thr thr thr  -   -   -  ser lys  -  ala lys his  -  asp lys ala lys asp ser ala 701
```

Table 11. The homology of the bovine calpastatin PCR cDNA at the nucleotide and amino acid sequence level to the corresponding regions in the pig, human and rabbit calpastatin cDNAs.

Species	Corresponding region to the Bovine calpastatin cDNA sequence (bp)	Corresponding region to the Bovine calpastatin primary sequence (aa)	Homology to Bovine calpastatin Nucleotide sequence (%)	Homology to Bovine calpastatin Primary sequence (%)
pig	1626-2070	543-690	82.2	73.3
human	1521-1965	508-655	78.2	74.6
rabbit	1638-2085	547-695	72.2	66.0

Within the calpastatin sequence there are regions that are conserved between the four domains and across species (56,59-61). Each domain has a central inhibitory consensus sequence, which has been shown to be essential for inhibitory activity, as well as two α-helical regions either side suggested to be involved in stabilizing the inhibitory sequence in the tertiary structure (59), as described in section 2.2.1.. These three regions have been identified in all the cDNAs so far isolated (59,61). Using the positions of the α–helical and central consensus regions proposed in other species they were identified in the partial amino acid sequence deduced from the bovine calpastatin cDNA . The regions are shown in Figure 35 as bold type above corresponding amino acid sequences from the other calpastatin cDNAs.

The two research groups, led by Murachi and Suzuki, have characterised the central inhibitory consensus sequences in the domain structure of calpastatin, as discussed in section 2.2.1.. The bovine central inhibitory consensus sequence is indicated in Figure 35, and can be written as;

L G E R D D T I P P K Y Q

based on the sequence suggested by Maki et al (59) Kawasaki et al (60) and Uemori et al (56). There is substitution from an arginine to a glutamine residue in the "well conserved" sequence (60) of;

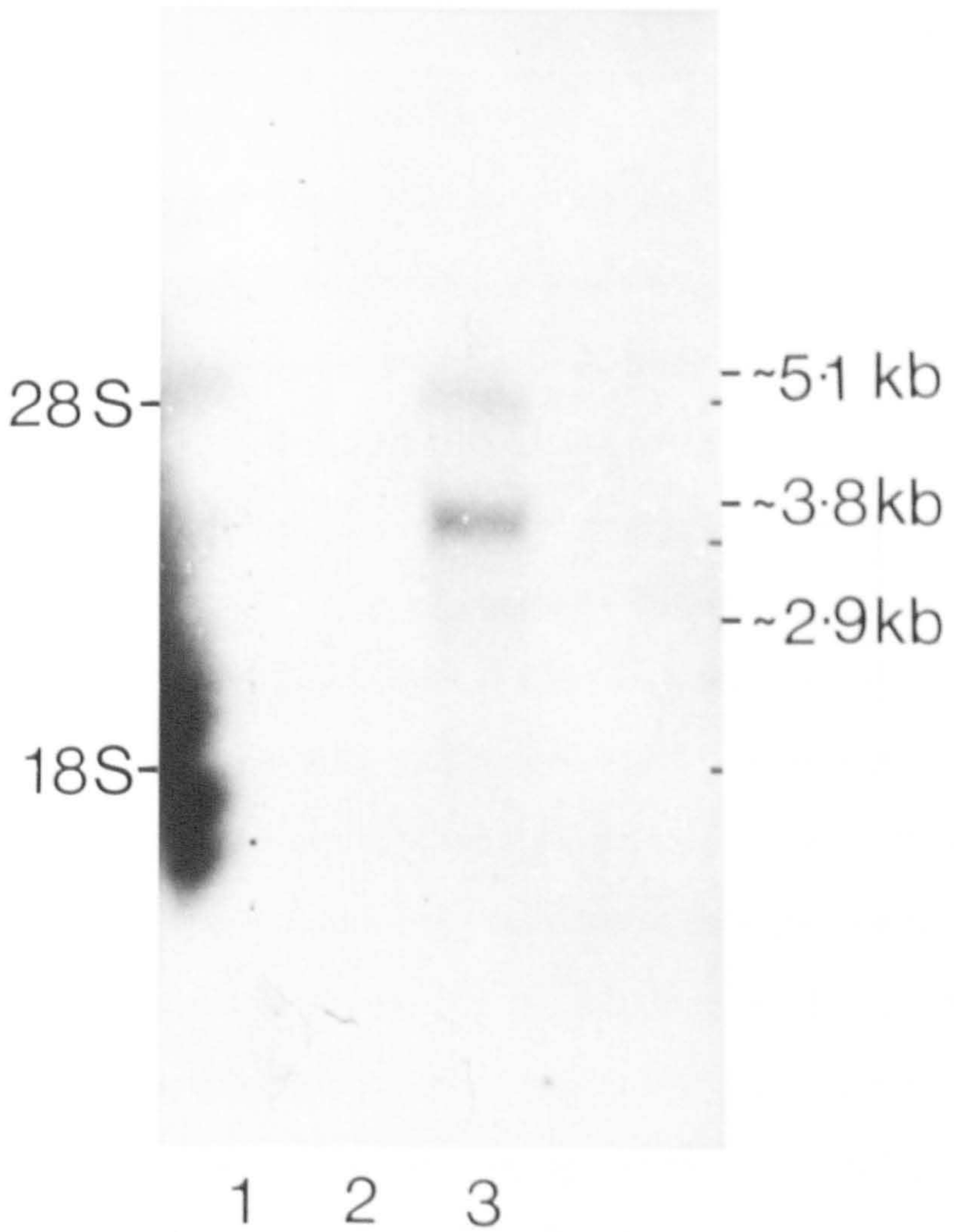
Thr Ile Pro Pro X Tyr Arg

From sequencing, in either direction in pG3p13 and pG3p21 there was no difference in this codon, it always appeared as CAA, a codon for glutamine. The implications of this amino acid substitution are considered in the discussion (section 5.1.).

The bovine calpastatin cDNA sequence was isolated from the 2 clones pG3p21/pG3p13, the plasmid inserts were mixed and used to probe a Northern blot

of bovine skeletal muscle poly(A)+ enriched RNA isolated by a single passage through a oligo-d(T) cellulose column. The result of the probe hybridization is shown in Figure 36. At low stringency washing, 60°C with 6xSSC, there were three hybridization bands visible. Two of the bands, at 5.1-5.0kb and 3.8-3.7kb, were clearly visible whilst a third was diffuse between 2.9-2.6kb. Increasing the stringency of washing up to 0.1xSSC plus 0.1% (w/v) SDS at 65°C left the two intense bands, but there was still a very faint but detectable region of hybridization at 2.9-2.6kb. Although the signal at 5.1-5.0kb overlapped the region of any rRNA in the poly(A)+ RNA sample the hybridization is strong even at high stringency washing, suggesting a specific hybridization product was present.

Figure 36: Northern blot of bovine skeletal muscle poly(A)+ RNA probed with bovine calpastatin PCR cDNA. Lane 1 and 2, Hind III lambda markers 4µg and 2µg respectively, lane 3, 9µg poly(A)+ RNA. Hybridized at 55°C and washed to a stringency of 65°C with 0.1xSSC plus 0.1% (w/v) SDS. The locations of the 28S and 18S ribosomal RNAs are marked.



4.2. Beta-Agonist Induced Skeletal Muscle Hypertrophy: Examination of the Effects of the Agonist Cimaterol on Skeletal Muscle mRNA with Particular Reference to the Calpain System.

An examination of the effects of β-agonists on the mRNA expression for the components of the skeletal muscle calpain system was made using the cDNA probes whose application in Northern blot analysis had been developed as described in the previous section. The samples used were provided from a bovine β-agonist growth trial using the agonist cimaterol. The experiment was carried out by Dr J.Dawson and I acknowledge her help in kindly making the muscle samples available for analysis as well as providing the details of the growth data which are described briefly below.

The trial was carried out on Friesian steers which were fed a 70% dry grass 30% barley pellet diet from 9 to 12 weeks of age. At 12 weeks the animals were divided equally into control (n=6) and treated groups (n=6) each group being of similar average starting weight. Both groups received the original diet but the treated group's included the β-agonist cimaterol at 1.5ppm. After 16 weeks the animals were slaughtered, the muscle samples dissected and frozen immediately in liquid nitrogen. Samples analysed were from five control and six cimaterol treated animals as one of the control animals died during the course of the experiment; however this did not alter similarity of the average starting weight of the two groups of steers (Table 12). Frozen muscle samples were stored at -40°C for up to 4 months until required for extraction of total RNA and assessment of enzyme activity.

The Longissimus dorsi (L.dorsi) muscle was examined for the effects of β-agonists on the expression of calpain system components mRNA. Previous studies had shown it to be a responding muscle to the growth promoting effects of β-agonists, which also had been linked to the changes seen in the activities on the calpain system in lambs (114), see section 2.4.0.. The effects of β-agonists on the calpain system had also been confirmed in an earlier steer β-agonist growth trial

Table 12. The effects of cimaterol treatment on the weight gain and longissimus dorsi characteristics in Friesian steers.

	Control (n=5)	Cimaterol (n=6)	SED	P
Starting body wt. (kg)	79.8 ± 4.8	79.9 ± 5.5	4.29	> 0.05
Final body wt.(kg)	200.7 ± 6.7	226.7 ± 8.8	11.41	< 0.05
Longissimus dorsi:				
Wet wt (g)	2050 ± 67	2817 ± 183	211	< 0.01
N (mg/kg wet wt)	35.88 ± 0.30	36.83 ± 0.24	0.38	< 0.05
Wet wt /unit empty body wt (g/kg empty body wt)	13.35 ± 0.32	15.59 ± 0.42	0.55	< 0.01

carried out within our laboratories (unpublished observations), see section 5.3.1. and Table 28.

The cimaterol treated animals had a significantly greater live weight (LW) at the end of the trial than the controls, Table 12. From the growth data it was apparent that the cimaterol induced skeletal muscle hypertrophy in the trial, Table 12. The L.dorsi was significantly heavier and was proportionally a larger part of body weight in the treated animals. The greater nitrogen content (N) of L.dorsi in the cimaterol treated animals reflects a increased protein accretion, a characteristic of β-agonist induced muscle hypertrophy.

The observations described above indicated that the cimaterol had induced muscle hypertrophy in bovine L.dorsi. The following sections describe the examination of the activities of the calpains and calpastatin, isolation of total RNA from the L.dorsi samples and the subsequent analysis for the effects of cimaterol on the expression of certain mRNAs.

4.2.1. Calpain and Calpastatin Activity.

L.dorsi muscle samples were ground to a powder under liquid nitrogen. Part of the sample was used for the extraction of total RNA and approximately 2g taken for the assessment of calpain and calpastatin activity. The calpain and calpastatin activity was isolated using the modified method involving phenyl-sepharose chromatography followed by FPLC on a Mono-Q anion exchange column (section 3.1.). Calpain and calpastatin activities were determined from the chromatographically separated enzymes and inhibitor (section 3.1.).

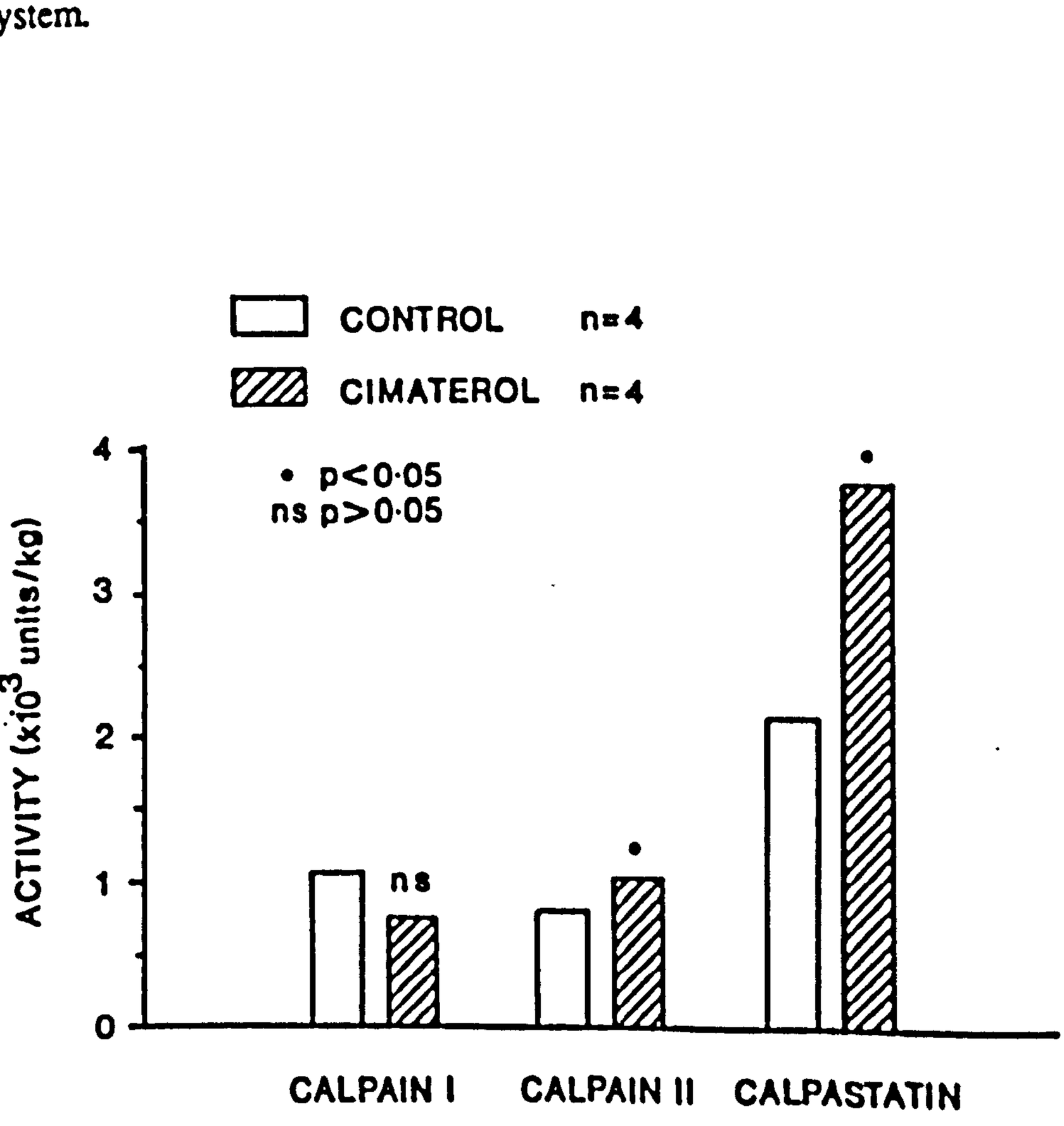
The isolation and the tests for calpain and calpastatin activity tests were carried out by Mrs Y.Lasslett whose help I gratefully acknowledge.

The effect of the β-adrenergic agonist, cimaterol, treatment on the calpain system in L.dorsi is shown in Table 13 and Figure 37. There was a statistically significant increase in calpain II and calpastatin activity with a decrease in calpain I which was not significant. This followed the trends seen in trials carried out previously in our laboratories on the effects of β-agonists in steers (unpublished observations) and lambs (114).

Table 13. The effect of cimaterol treatment on the calpain system activities in bovine L.dorsi .

	Activity (U/kg muscle)			%	
	Control (n=4)	Cimaterol (n=4)	SED	increase	P
Calpain I	1073	767	198	-29	> 0.05
Calpain II	813	1036	64	28	< 0.05
Calpastatin	2138	3759	636	76	< 0.05

Figure 37: The effect of cimaterol treatment on the activity of the bovine L.dorsi calpain system.



4.2.2. Extractable Total RNA.

Total RNA was extracted from 8g of ground musole powder from cimaterol treated and control L.dorsi samples (section 3.2.1.). The resulting total RNA was dissolved in water and the concentration evaluated by its absorbance at 260nm (section 3.2.5.). The quantity of total RNA extracted is shown in Table 14.

There is less extractable intact total RNA present in the cimaterol samples than the controls but it was not a statistically significant difference.

To give some indication of the quality of the RNA extracted three samples of each group were subjected to denaturing agarose gel electrophoresis (section 3.2.7.(i)). As can be seen in Figure 38 the samples appeared to be of good quality, since the 18S and 28S rRNA bands were clearly visible. There were definite bands between the rRNAs which could have been large quantities of particular RNA species.

The negative of the photograph in Figure 38 was scanned on the Ultrascan XL^{TM} Laser Densitometer and the scanning data analysed on the Gelscan XL^{TM} Software Package (LKB) (section 3.4.14.). The scans were used to try and assess if there was any change in the expression of the rRNA in the skeletal muscle total RNA extracted from the treated and untreated animals.

The mRNA makes up a small proportion of the total RNA, the cytoplasmic mRNA being approximately 3-4%. The rRNA is by far the greatest constituent of total RNA being up to 75% (215). By assessing the area under the clearly distinguishable 18S and 28S peaks above background using a manually selected baseline only a small specific part of the rRNA is being examined for changes (Figure 39). However, as the total RNA concentration did not significantly alter between control and cimaterol-treated L.dorsi then the combined 18S and 28S peak areas did give an indication of the relative expression of these RNA species to others in the total RNA of control and treated animals. An increase in the 18S and 28S combined peak area would have been an indication that there was augmented expression of the RNA making up the ribosomes which may have implications on the capability for enhanced

Table 14. The effect of cimaterol treatment on the quantity of total RNA extracted from bovine L.dorsi .

	Control (n=5)	Cimaterol (n=6)	SED	P
Total RNA (µg/g muscle)	172 ± 7	152 ± 10	12.8	> 0.05

(values are the mean ± SEM)

Figure 38: Glyoxal denaturing 1% agarose gel electrophoresis of total RNA extracted from the cimaterol treated and control bovine L.dorsi. The Hind III lambda DNA (0.5µg) lane is marked M and the cimaterol samples are indicated by 'X'. The ribosomal RNAs 28S and 18S are shown.

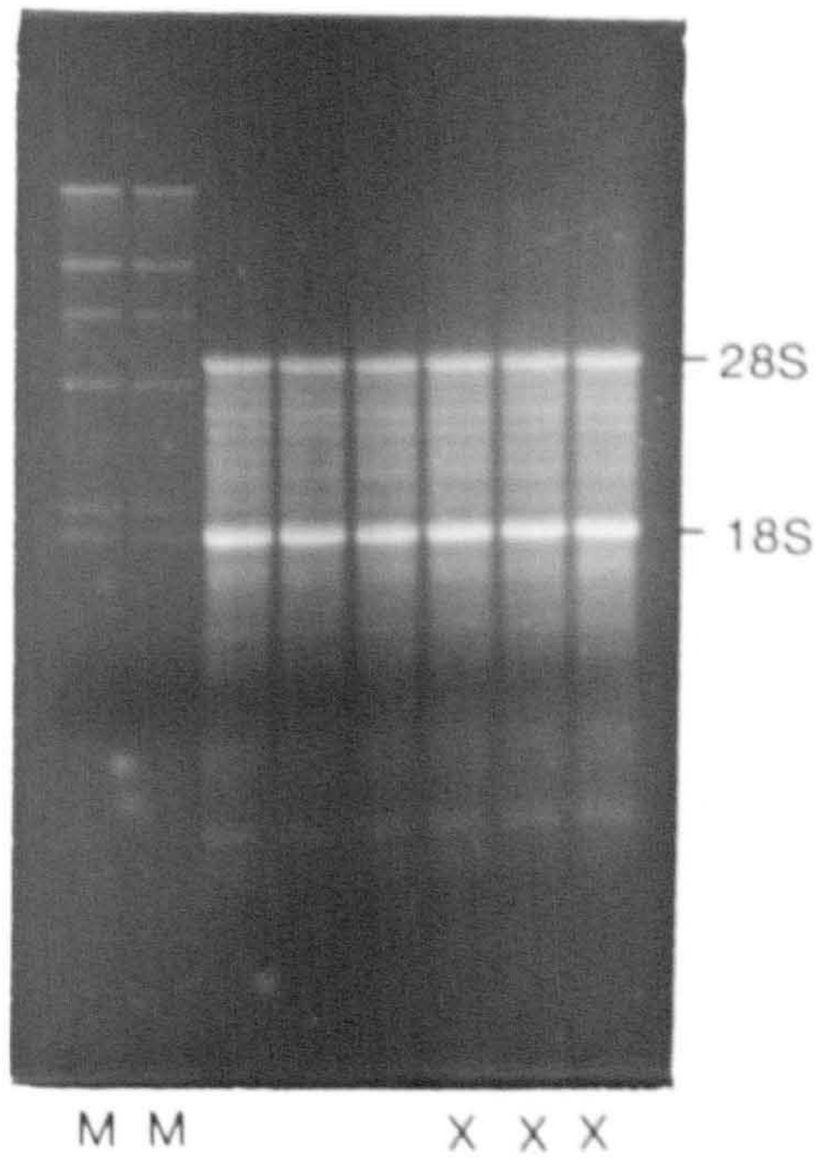

28S
18S
M M
X X X

Table 14. The effect of cimaterol treatment on the quantity of total RNA extracted from bovine L.dorsi .

	Control (n=5)	Cimaterol (n=6)	SED	P
Total RNA (µg/g muscle)	172 ± 7	152 ± 10	12.8	> 0.05

(values are the mean ± SEM)

Figure 38: Glyoxal denaturing 1% agarose gel electrophoresis of total RNA extracted from the cimaterol treated and control bovine L.dorsi. The Hind III lambda DNA (0.5µg) lane is marked M and the cimaterol samples are indicated by 'X'. The ribosomal RNAs 28S and 18S are shown.

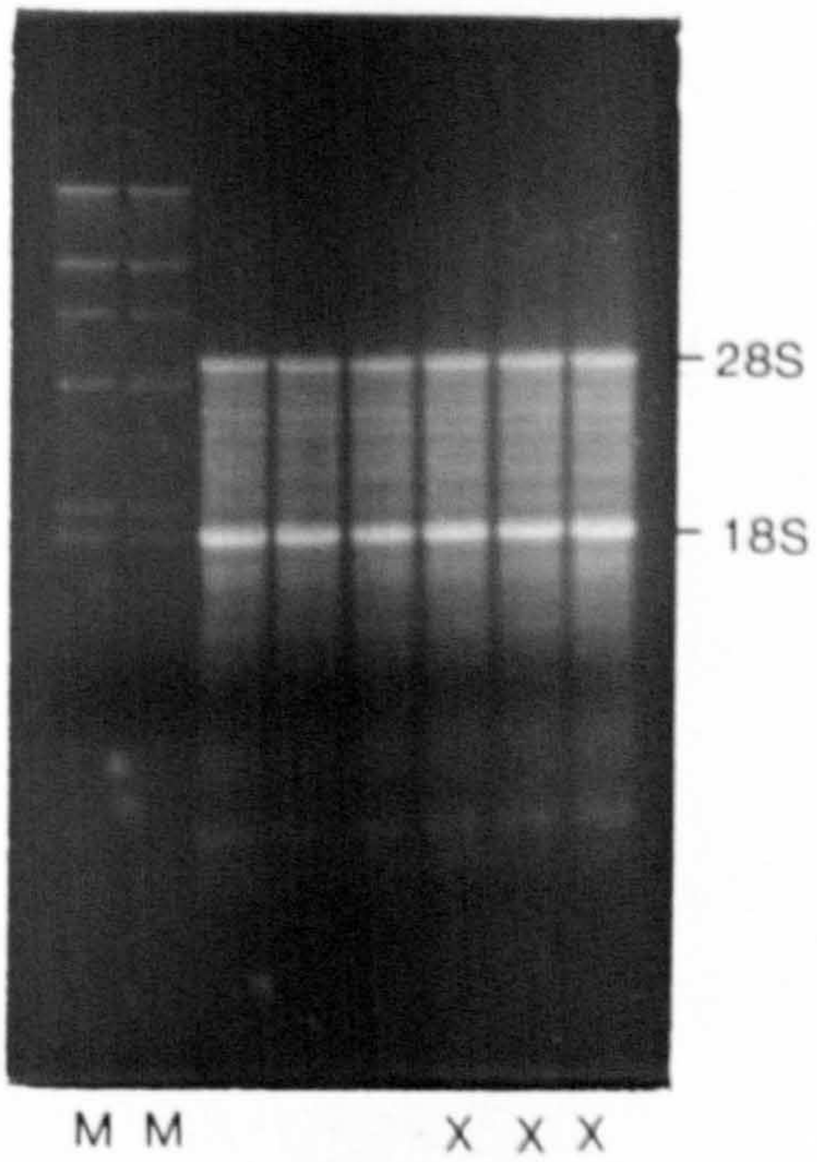


Figure 39: A representative scanning densitometry trace produced from the denaturing gel in Figure 38. The trace shows the position of the 28S and 18S rRNA peaks and the selected baseline for peak evaluation.

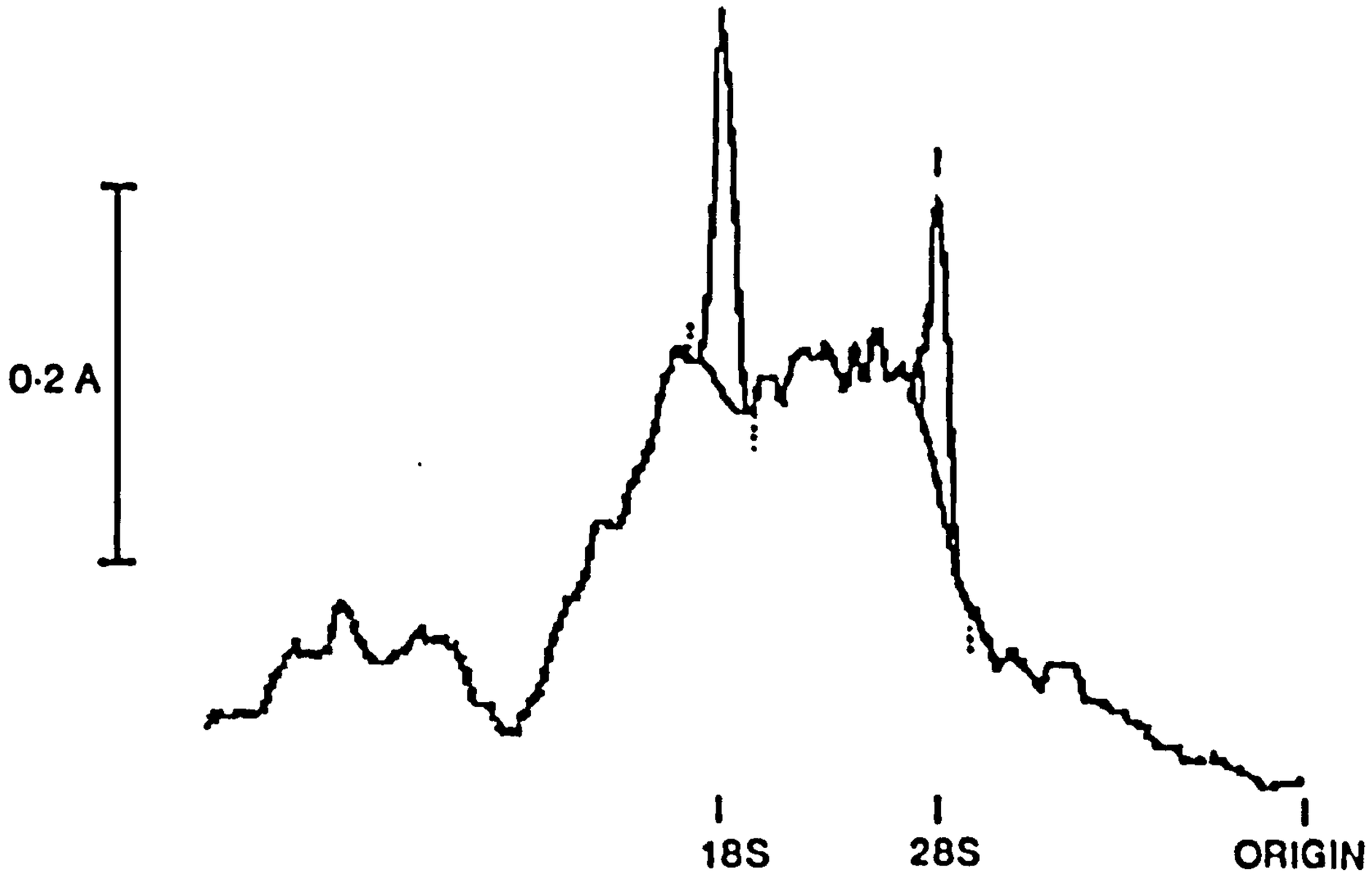


Table 15. The effect of cimaterol on the amount of 18S and 28S rRNA species present in total RNA .

	Densitometry signal area under peak (Arbitrary units)		SED	P
rRNA	Control (n=3)	Cimaterol (n=3)		
18S	0.188 ± 0.007	0.171 ± 0.002	/	/
28S	0.153 ± 0.002	0.159 ± 0.003	/	/
Combined ribosomal	0.341 ± 0.009	0.330 ± 0.001	8.8×10^{-3}	> 0.05

(values are the mean ± SEM)

translational capacity.

The analysis of the rRNA content of the muscles by this method gave a semi-quantitative assessment. Table 15 displays the values for the peak areas calculated by integrating the area under 18S and 28S peaks. From the combined areas of the two peaks there was no significant difference between the cimaterol and control samples.

4.2.3. In Vitro Translation of Total RNA.

The bovine skeletal muscle total RNA from the cimaterol and control samples was subjected to in vitro translation using the rabbit reticulocyte lysate system (section 3.2.10.).

Initially in vitro translation was carried out using a total RNA concentration of 2-4µg per translation. All the samples tested showed incorporation of ^{35}S into TCA precipitable protein, as assessed by the filter dotting method using 2µl of the translation mix at a level that was 4 to 6 times that of the blank. The remaining volume of the samples were run out on a 10% SDS-PAGE (section 3.2.11). The autoradiograph of the gel showed protein products with ^{35}S incorporated (data not shown). This indicated that the RNA isolated from the bovine L.dorsi was intact.

There was a significant increase in the ^{35}S incorporated into TCA precipitable protein for the translations carried out on total RNA from the cimaterol treated animals (Table 16A). However when the values obtained were expressed per µg of total RNA the difference in the incorporation was not significant (Table 16B). It was not known whether the concentrations of total RNA used in the experiment were saturating.

In order to try and confirm whether the total RNA isolated from the cimaterol treated bovine L.dorsi had the capacity for enhanced in vitro translation capability the translations were repeated using a non-saturating concentration of total RNA, which was determined by a dilution assay of a sample from a cimaterol treated animal (Table 17). It appeared that a total RNA concentration of 1µg per translation was on the

Table 16: The incorporation of L-[^{35}S]-methionine into TCA precipitable protein of cimaterol treated and control L.dorsi total RNA subjected to in vitro translation.

	Control (n=4)	Cimaterol (n=4)	SED	P
A. ^{35}S Incorporation (cpm/2µl)	16028 ± 1726	21557 ± 837	1918	< 0.05
B. ^{35}S Incorporation (cpm/2µl/µg)	5502 ± 1348	7162 ± 1142	1766	> 0.05

Table 17: The incorporation of L-[^{35}S]-methionine into TCA precipitable protein generated by in vitro translation with increasing concentration of total RNA present.

Dilution	RNA (µg)	Incorporation of ^{35}S (cpm/2µl)	Activity loaded onto gel (cpm)
0	0	2399	9596
1/8	0.46	9012	36048
1/4	0.93	13083	52332
1/2	1.85	23276	93104
1	3.70	25574	102296

Figure 40: The change in L-[^{35}S] methionine incorporation into TCA precipitable protein by increasing the concentration of total RNA present in the <u>in vitro</u> translation mix (data from Table 17).

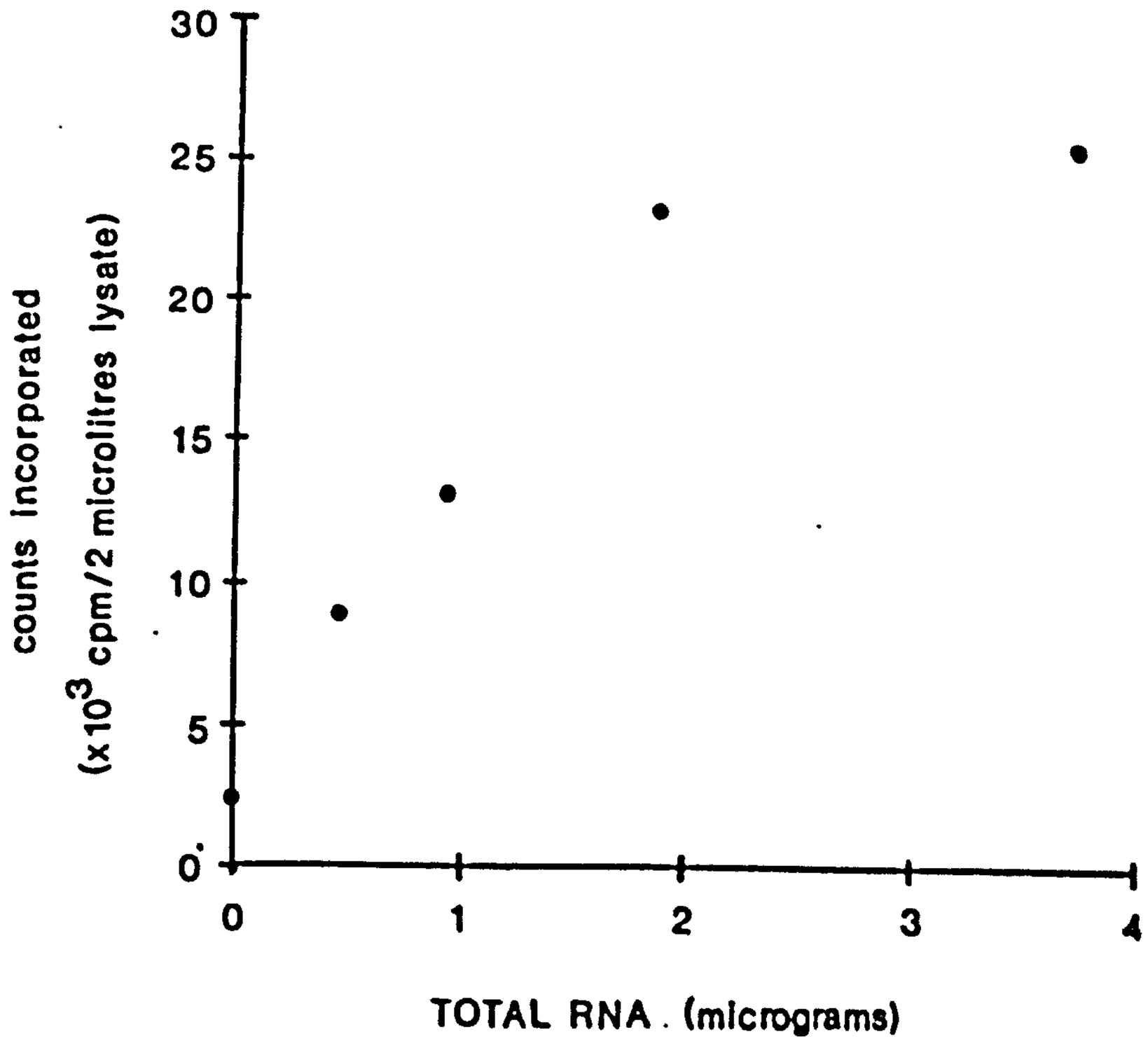
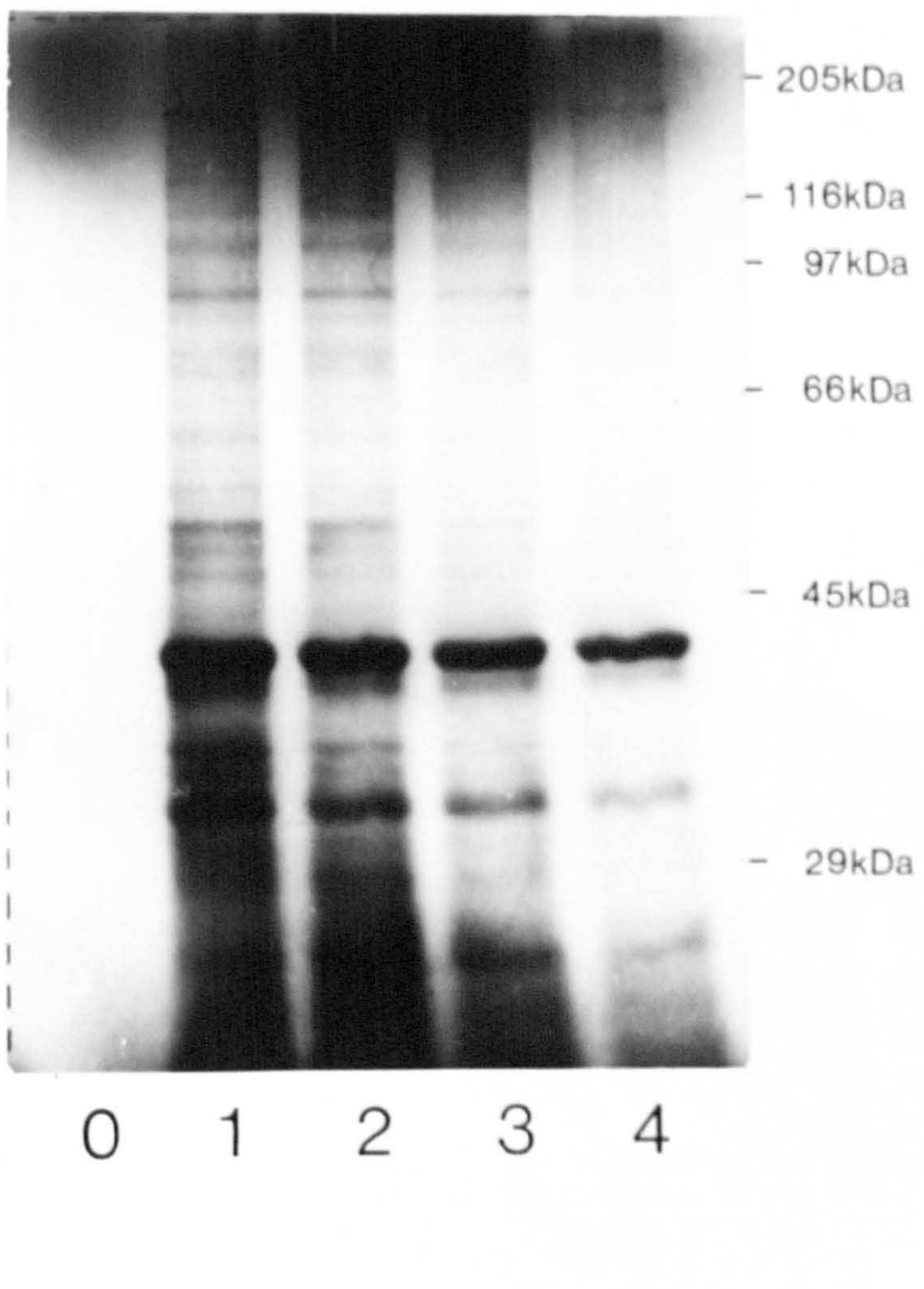


Figure 41: A 10% SDS-PAGE gel of the in vitro translation products generated from total RNA at various dilutions. The samples loaded are the in vitro translation products from 0, 3.7, 1.85, 0.93 and 0.46µg L.dorsi total RNA, lanes 0 to 4 respectively. The quantity of activity loaded is shown in Table 17. The positions of nonradioactive markers are indicated.



linear part of the total RNA translation saturation curve (Figure 40). The remaining translation products, as indicated in Table 17, were subjected to SDS-PAGE (Figure 41). The autoradiograph confirmed that there was incorporation of ^{35}S-methionine into protein as well as showing the level of activity required which gave clearly visible protein products on autoradiographed SDS-PAGE, which was in the range of 90-100x10^3cpm per lane.

Using 1µg total RNA per translation an assessment of the translation capability of the cimaterol and control samples was made. The stock of total RNA used in the first translation was diluted to 1µg/µl then the concentration redetermined by absorbance at 260nm. The translation was carried out as described in section 3.2.10.. There was no significant difference in the incorporation into TCA precipitable protein per µg of total RNA present between the control and cimaterol samples at nonsaturating total RNA concentrations (Table 18).

The in vitro translations were repeated using 2.0-2.5µg of total RNA per translation (Table 19), concentrations which were believed to be near saturating, see Figure 40. There was no significant difference between the samples although the cimaterol samples seemed to show a higher incorporation per µg than the controls. From this translation the equivalent of 90x10^3 cpm was taken from each sample and subjected to SDS-PAGE. The resulting autoradiographs are shown in Figure 42.

There appeared to be no major changes in the protein produced from the total RNA of the cimaterol treated and nontreated muscle total RNA samples. The probable translation products of actin and myosin can be seen. At the bottom of the gel are the smaller muscle proteins, possibly the myosin light chains, which do show some slight size shifts between the controls and cimaterol-treated groups in some of the samples.

The in vitro translations indicated that the mRNA was intact. The proteins encoded by the mRNA present were not appreciably different in this qualitative estimation nor was there an increase in the protein of particular transcripts between the treated and control animals.

Table 18. The effects of cimaterol on the translational capability of bovine L.dorsi total RNA determined at 'nonsaturating' concentrations.

	Control (n=3)	Cimaterol (n=3)	SED	P
^{35}S Incorporation (cpm/2µl/µg)	12440 ± 3430	12470 ± 3680	5030	> 0.05

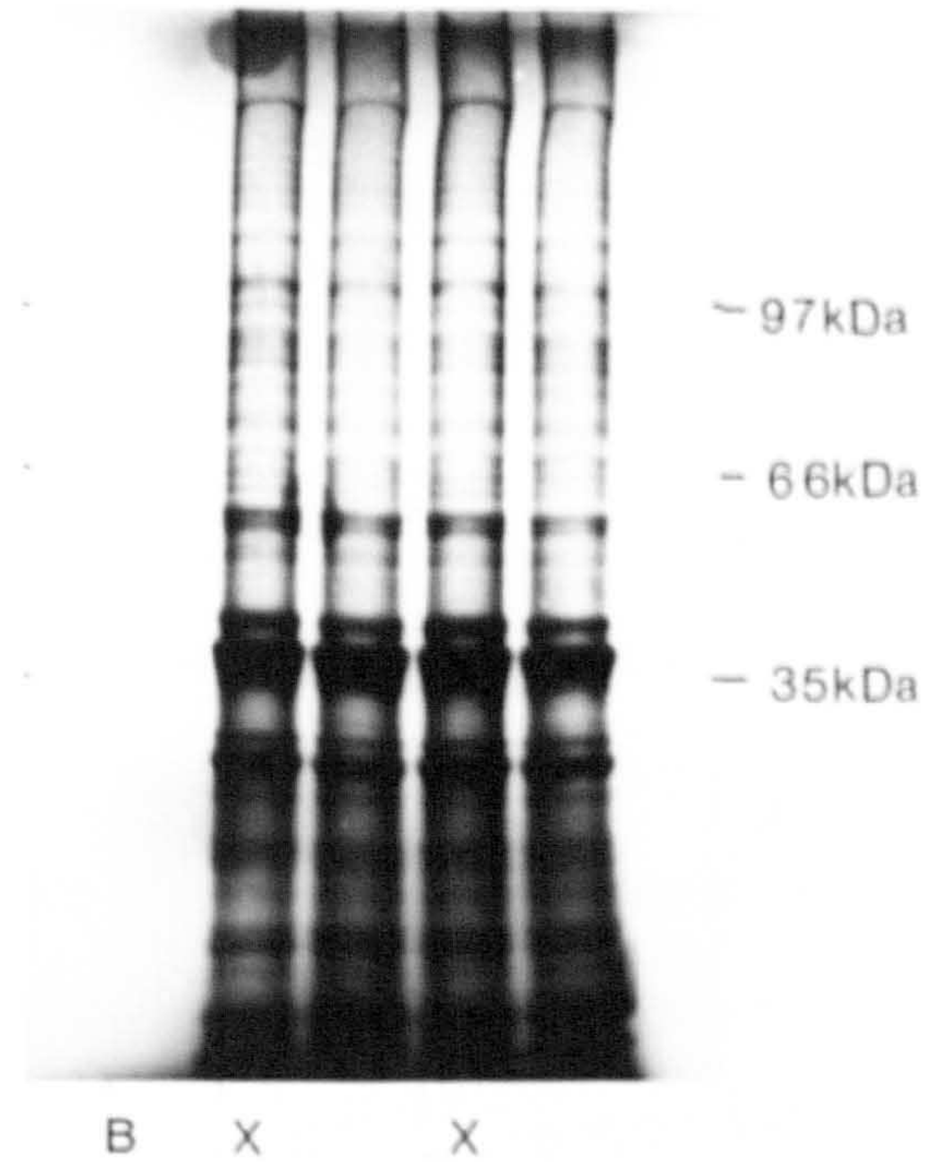
(values are the mean ± SEM)

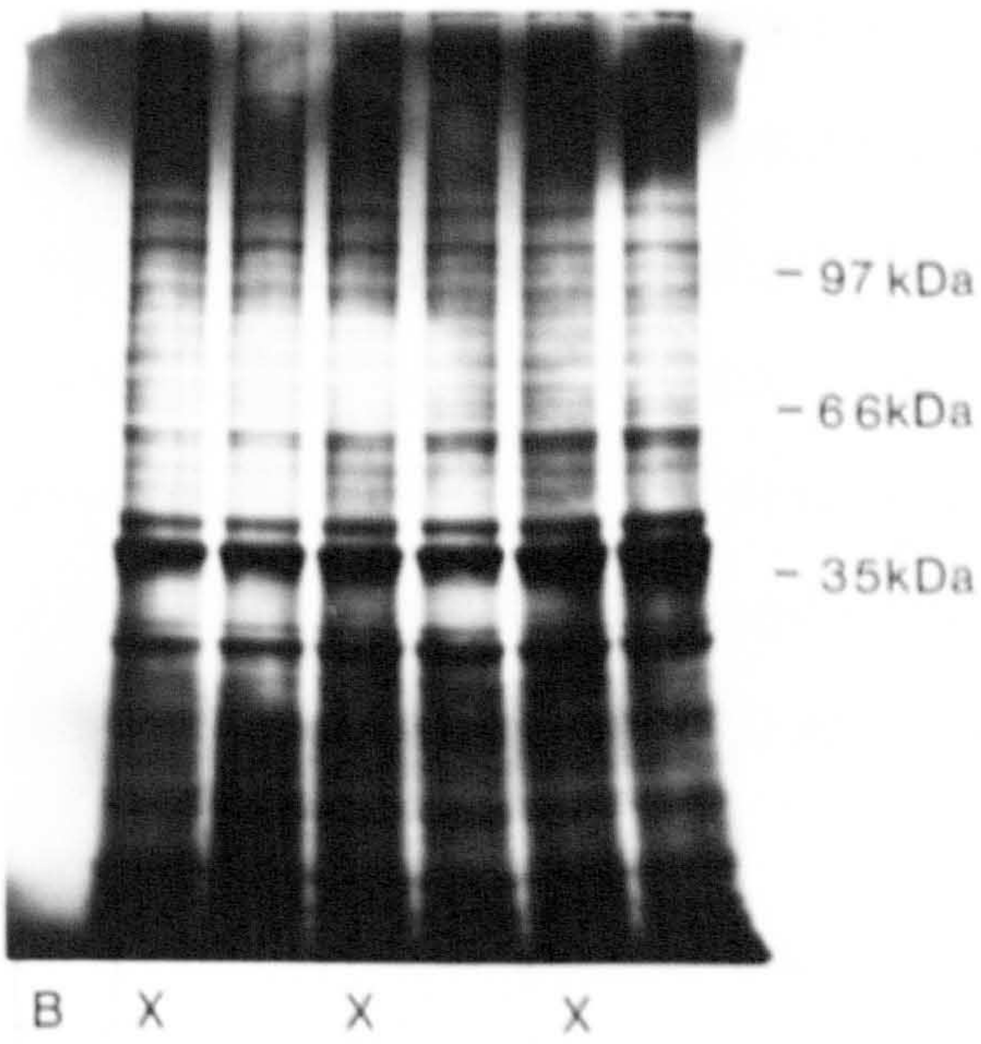
Table 19.The effects of cimaterol on the incorporation of L-[^{35}S]methionine into TCA precipitable protein in the <u>in vitro</u> translation at 'saturating' levels of bovine L.dorsi total RNA (2.0-2.5µg).

	Control (n=5)	Cimaterol (n=5)	SED	P
^{35}S Incorporation (cpm/2µl/µg)	9620 ± 1050	12730 ± 1040	1480	> 0.05

(values are the mean ± SEM)

Figure 42: A 10% SDS-PAGE of the in vitro translation products generated by 2.0-2.5µg of bovine L.dorsi total RNA from cimaterol treated and control animals. The cimaterol samples are marked with 'X' and the blank is indicated by the letter B. The number of samples dictated that two gels were used for analysis of the L-[^{35}S] methionine labelled in vitro translation products. The positions of the nonradioactive markers are shown.





4.2.4. Determination of Changes in Specific Bovine Skeletal Muscle mRNA.

Northern blot analysis

Using mouse α-actin cDNA and chicken myosin light chain 2 (MLC2) cDNA (appendix A) skeletal muscle total RNA from the cimaterol treated and control animals was probed to assess whether there were changes in the equivalent bovine mRNAs. The two cDNA probes had been used with success on bovine total and poly(A)+ RNA; they had hybridized to a specific mRNA species in each case under relatively high stringency washing conditions 2xSSC plus 0.1% (w/v) SDS at 65°C (section 4.1.1. and Figure 22 and 23).

A Northern blot of control and cimaterol treated total RNA (25μg) samples was probed with the mouse α-actin cDNA labelled by nick translation (section 3.4.9.(i)). The membrane was hybridized at 55°C and washed to a stringency of 0.1xSSC plus 0.1% (w/v) SDS at 65°C as shown in Figure 43. After scanning densitometry of the autoradiograph the peak areas of the trace produced was determined using the data analysis integration programme (3.4.14.). The scans of the autoradiograph and the results of the assessment of their peak area by integration are shown in Figure 44 and Table 20 respectively. There was an apparent statistically significant increase in the mRNA for α-actin in the cimaterol samples.

The α-actin cDNA was removed from the Northern blot (section 3.4.13.). The Northern blot was reprobed with the chicken MLC2 cDNA labelled by nick translation. Hybridization was carried out at 55°C and the membrane was washed at increasingly stringent conditions to 2xSSC plus 0.1% (w/v) SDS at 65°C. The autoradiograph, Figure 45, was scanned on the densitometer and the area under the peaks of the trace determined as for the α-actin probed Northern blot , as indicated in Table 21.

As with the α-actin cDNA probe hybridization experiment there was a significant increase in the mRNA transcripts for MLC2 in the skeletal muscles of the cimaterol treated animals.

Figure 43: A Northern blot of cimaterol treated and control bovine L.dorsi total RNA (25μg) probed with mouse α-actin cDNA. The hybridization and washing details are in the text. Positions of the ribosomal RNAs, 28S and 18S, are indicated and the cimaterol samples are marked with 'X'. The size of α-actin hybridization signal is indicated .

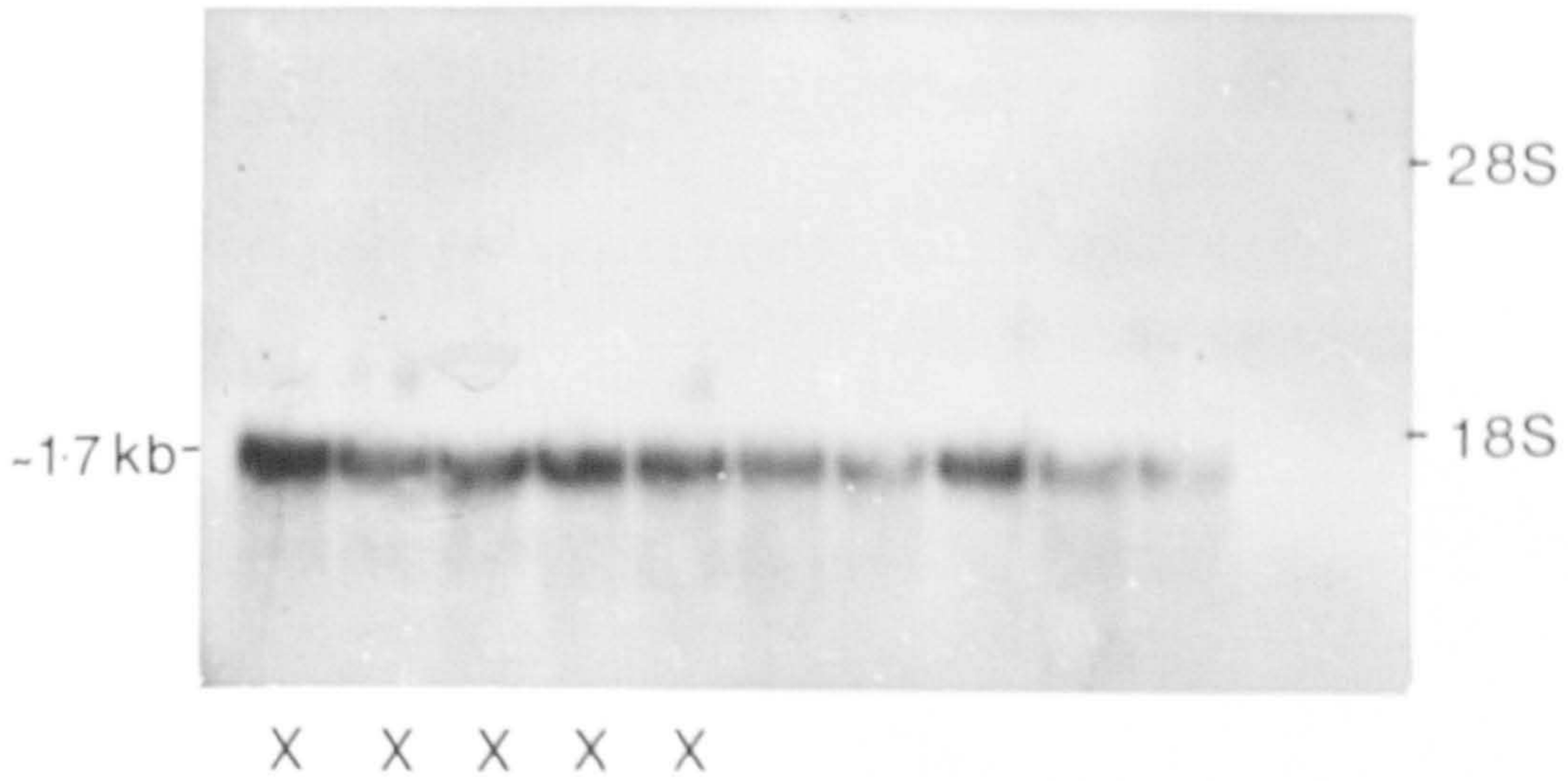


Figure 44. Scanning densitometer traces produced from the Northern blot (Figure 43) probed with mouse α-actin cDNA. Traces used for the assessment of the intensity of the hybridization signals (Table 20).

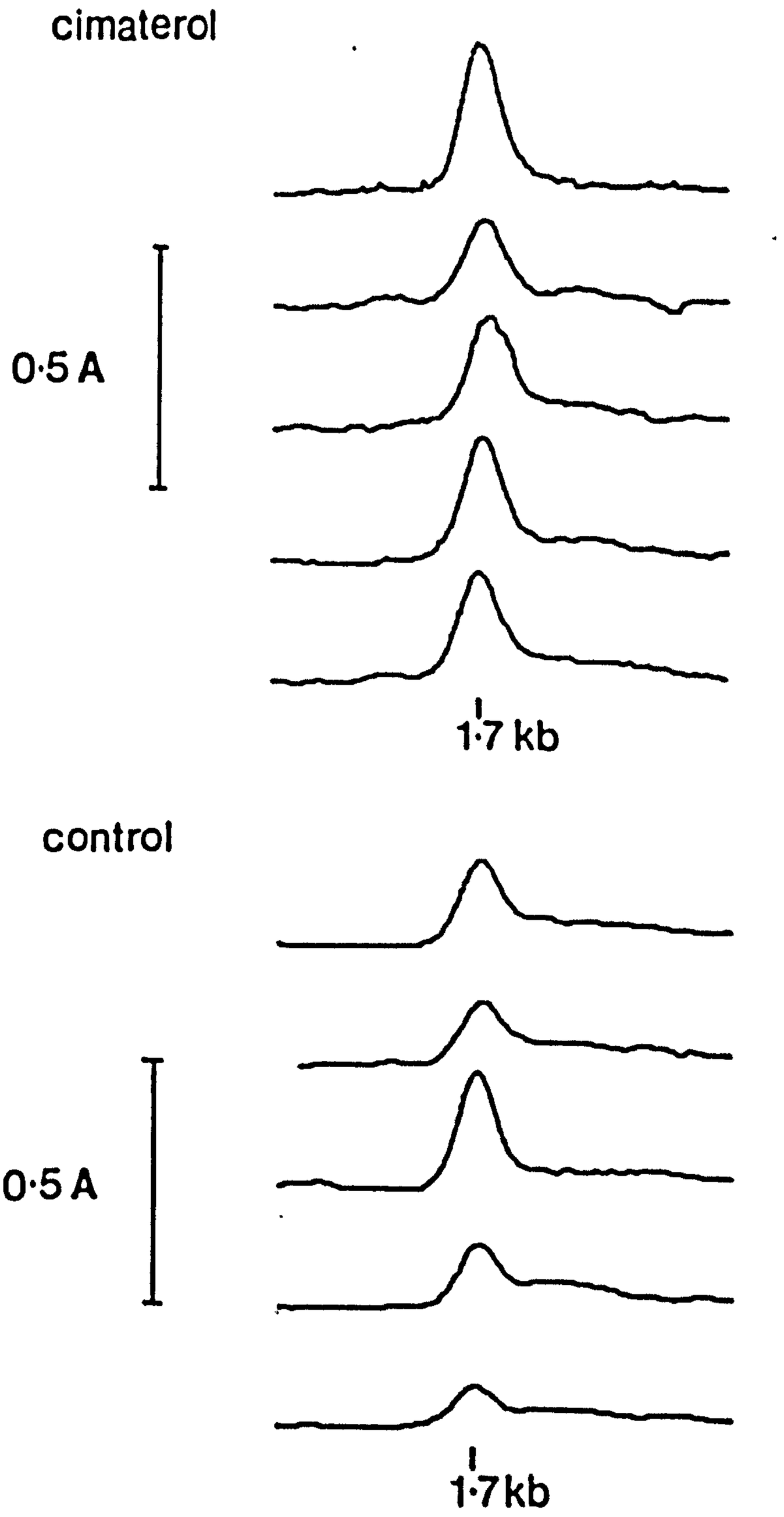


Table 20. The effect of cimaterol on the hybridization signal produced from the Northern blot (Figure 43) of bovine L.dorsi total RNA probed with mouse α-actin cDNA.

	Control (n=5)	Cimaterol (n=5)	SED	P
Area by Integration (Arbitrary units)	0.708 ± 0.140	1.164 ± 0.125	0.186	< 0.05

(values are the mean ± SEM)

Figure 45: The Northern blot of cimaterol treated and control bovine L.dorsi total RNA samples (25µg) probed with chicken MLC2 cDNA. The blot shown below was Figure 43 reprobed. Hybridization and washing details are in the text. Positions of the 28S and 18S ribosomal RNAs are shown and the cimaterol samples are marked by 'X'. The size of the hybridization signal for the MLC2 is indicated.

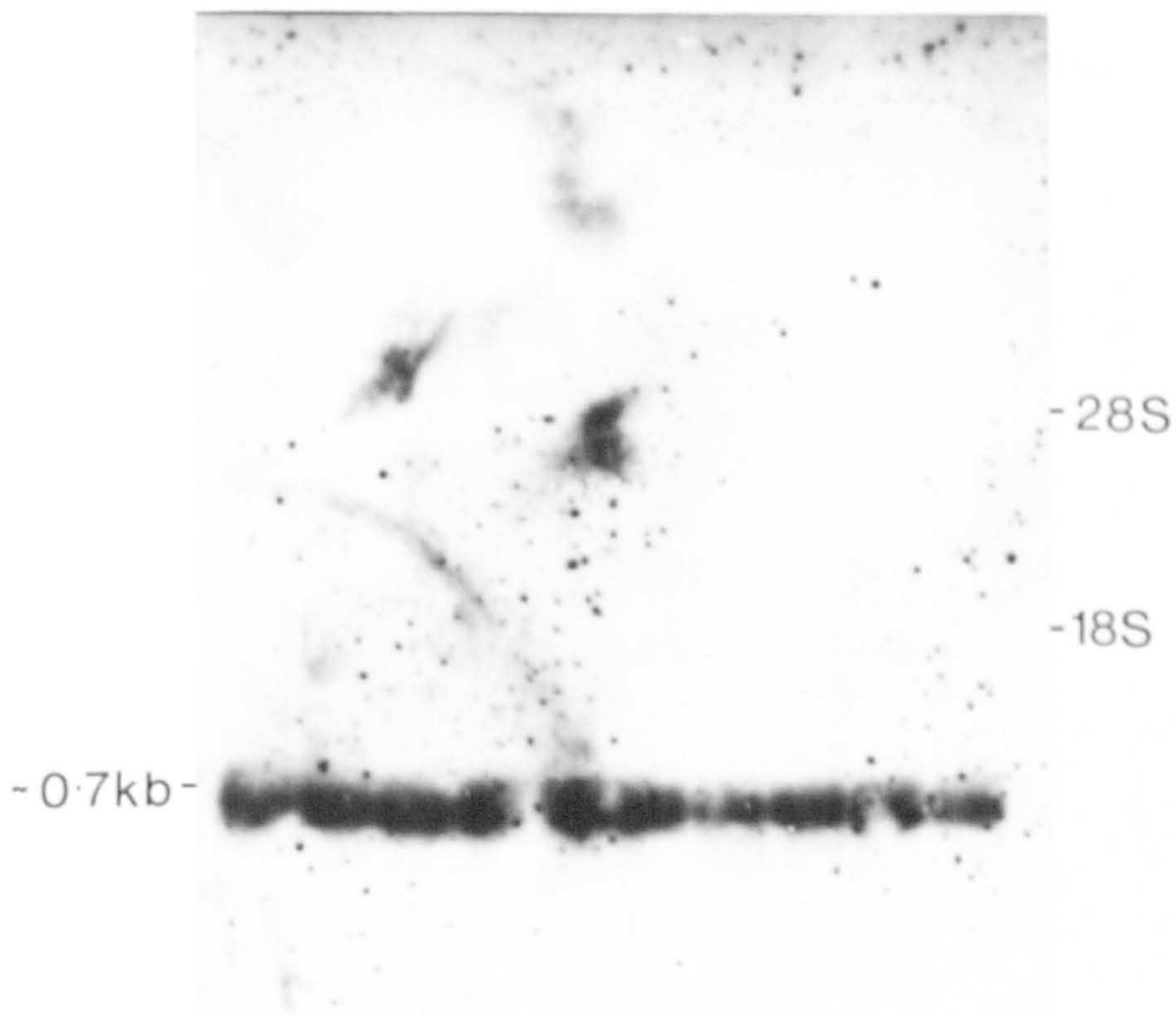


Table 21. The effect of cimaterol on the hybridization signal produced from the Northern blot (Figure 45) of bovine L.dorsi total RNA probed with chicken MLC2 cDNA.

	Control (n=5)	Cimaterol (n=5)	SED	P
Area by Integration (Arbitrary units)	3.172 ± 0.287	4.264 ± 0.364	0.464	< 0.05

(values are the mean ± SEM)

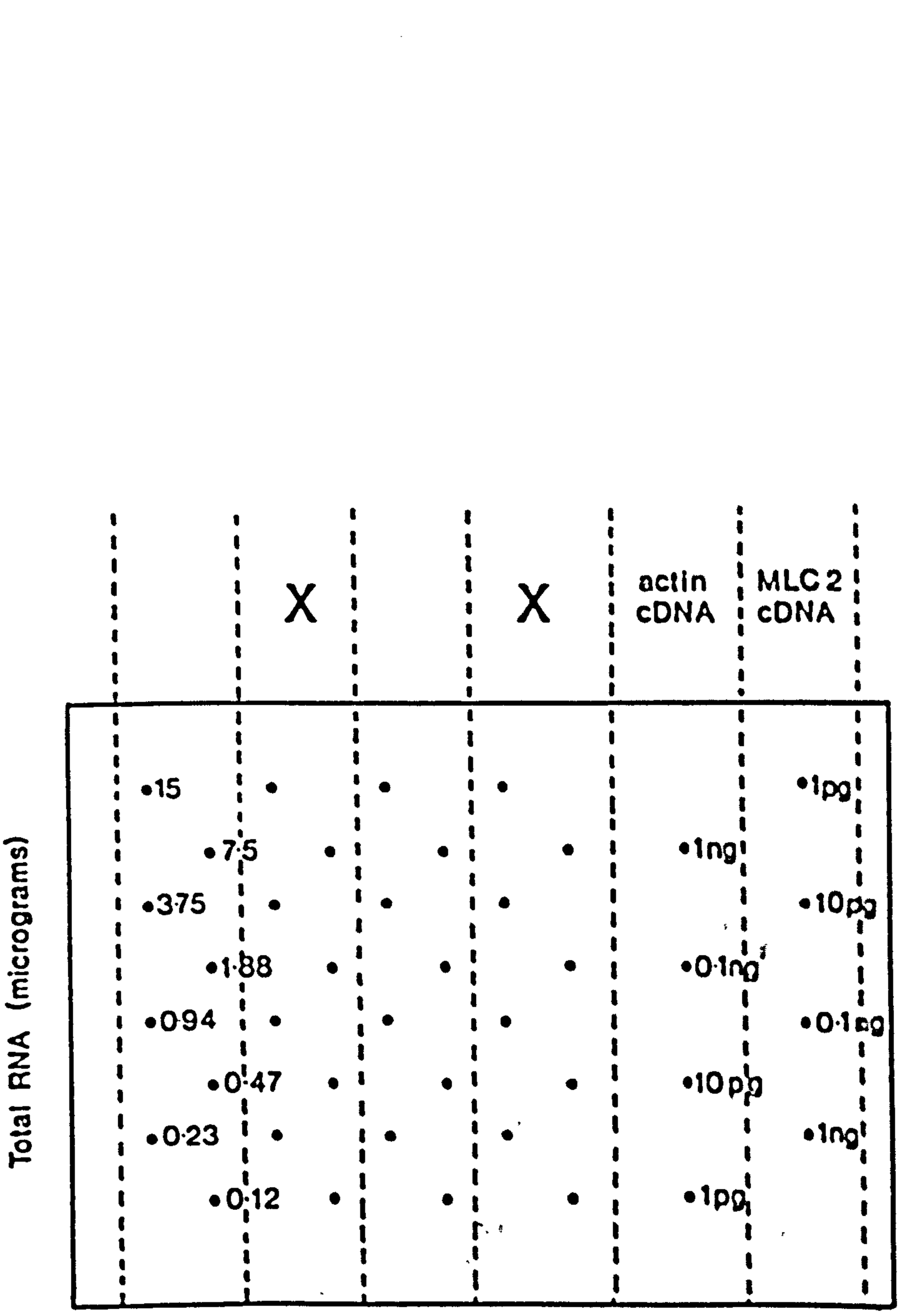
However from previous Northern blots the distribution of the samples and uneven transfer of the RNA had resulted in variation of hybridization signal on membranes. To confirm the changes seen in the Northern blots, slot and dot blots of total RNA were used.

Dot and slot blot analysis.

Serial half dilutions of total RNA were used in dot and slot blots using a formamide/formaldehyde denaturing system (section 3.2.9.). Two samples from each group were applied on to a dot blot apparatus in half dilutions from 15µg of total RNA. It was difficult to apply the 15µg to the dot area onto the Hybond-N membrane (Amersham) as the sample was too concentrated for the area to be loaded. The blot was probed with chicken MLC2 cDNA at 55°C and washed at increasing stringency to 2xSSC plus 0.1% (w/v) SDS at 65°C. The resulting autoradiograph is shown in Figure 46. Analysis of the dot blot scans was carried out by scanning densitometry of the autoradiograph and subsequent evaluation of resulting peak area by integration of the scan (Table 22). The 15µg sample was disregarded due to its inconsistent application onto the Hybond-N membrane.

The data in Table 22 was used to determine the relationship between the amount of total RNA in each dot and the area under the densitometer peak for each dot, Figure 47. The curve was only linear over a restricted range of total RNA loaded from 0 to 2µg above which the curve reached a plateaux. Linearity is achieved where the quantity of the sequence being probed on the dot is less than that of the concentration of probe present in the solution. From the linear part of the curve there apparently was no difference between the samples.

Figure 46: Dot blot of total RNA from cimaterol treated and control bovine L.dorsi probed with MLC2 cDNA. A loading plan for the quantity of total RNA present is shown along with positions of the controls dots of MLC2 and α-actin cDNA. The cimaterol samples are marked X. Hybridization and washing details are described in the text.



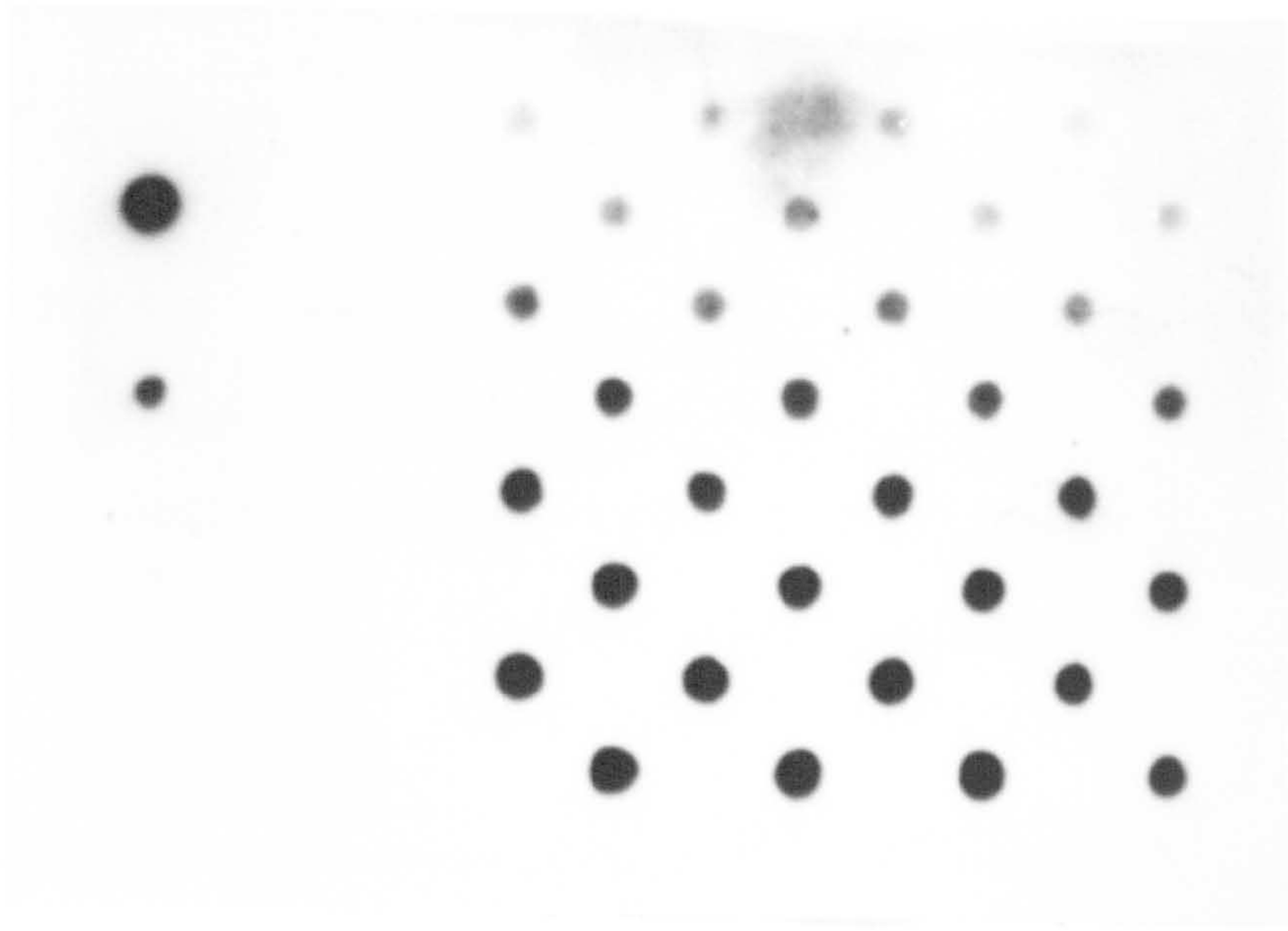


Figure 46: Dot blot of total RNA from cimaterol treated and control bovine L.dorsi probed with MLC2 cDNA. A loading plan for the quantity of total RNA present is shown along with positions of the controls dots of MLC2 and α-actin cDNA. The cimaterol samples are marked X. Hybridization and washing details are described in the text.

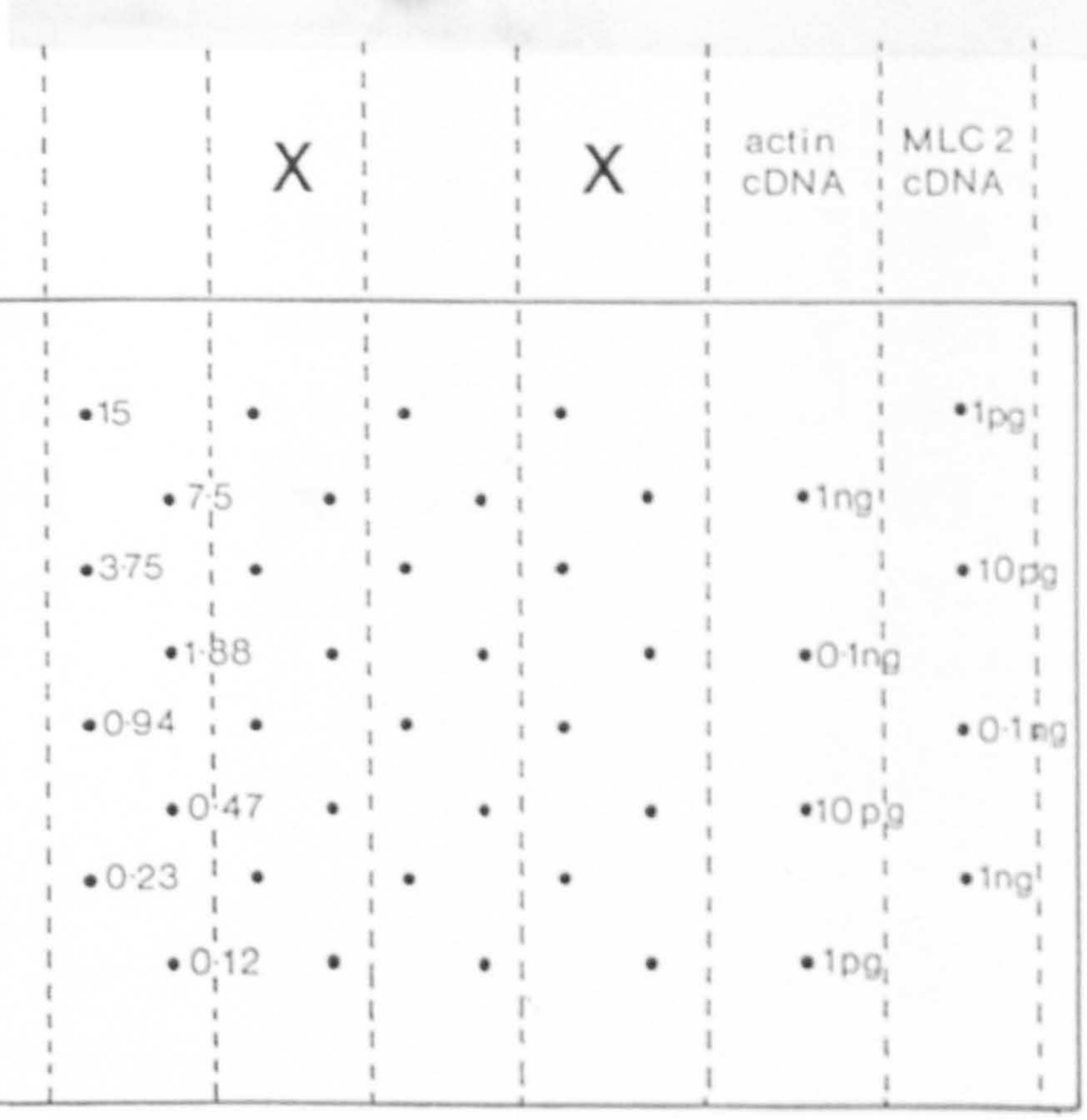
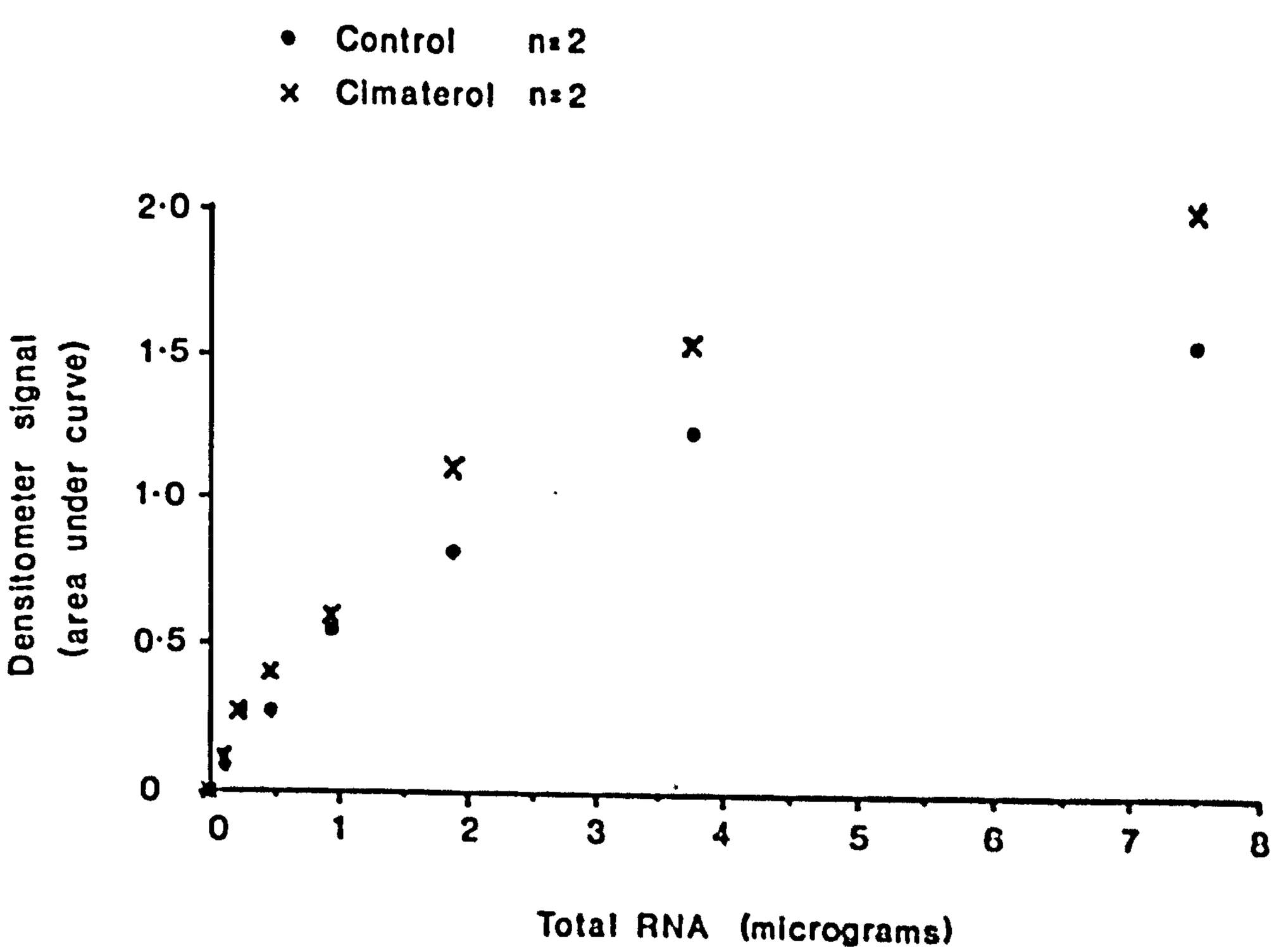


Table 22. Area under the absorbance peaks of the densitometer trace from the chicken MLC2 cDNA probed dot blot (Figure 46) of cimaterol treated and control bovine L.dorsi total RNA .

Dilution	RNA (µg)	Densitometer signal (Area under curve, Arbitrary units) Control (n=2)	Cimaterol (n=2)
1	7.50	1.552	2.008
1/2	3.75	1.236	1.537
1/4	1.88	0.882	1.102
1/8	0.94	0.547	0.591
1/16	0.47	0.276	0.404
1/32	0.23	0.262	0.274
1/64	0.12	0.095	0.109

(values are the mean)

Figure 47: The relationship between the total RNA present in each dot and the intensity of the hybridization signal for the MLC2 cDNA probed dot blot (Figure 46). The data is from Table 22.

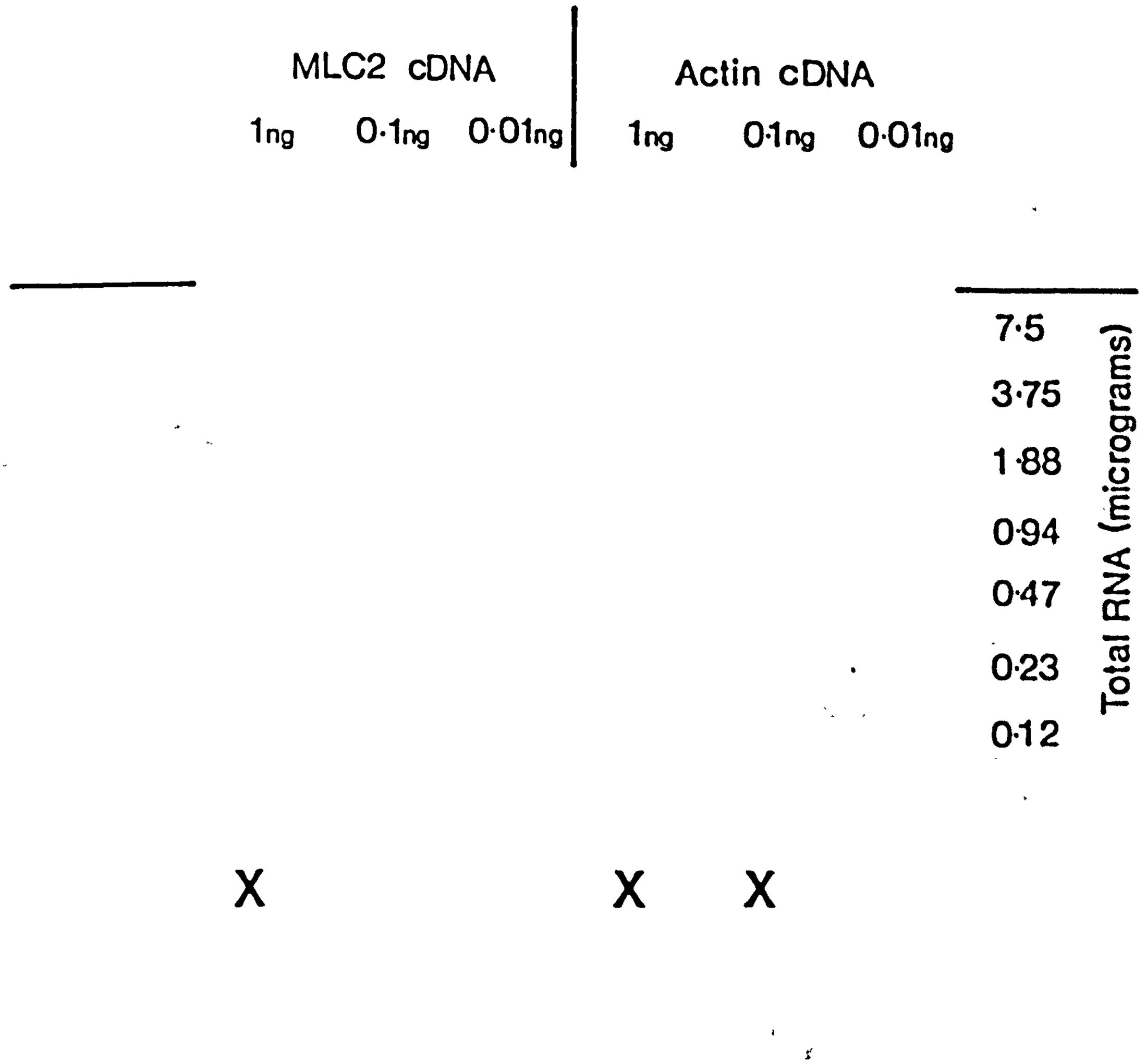


The analysis was repeated using slot blotting (section 3.2.9.) which gave more distinct sample loading and resulting autoradiograph pattern than the dot blot apparatus. Samples were loaded in a series of half dilutions from 7.5µg downwards. The membrane was probed with MLC2 cDNA at 55°C then washed to a stringency of 2xSSC plus 0.1% (w/v) SDS at 65°C. The resulting autoradiograph is shown in Figure 48.

The average area from the integration of the traces is shown in Table 23, the graph produced from the data is shown in Figure 49. However it was difficult with the parameters selected to obtain an integration value from the 7.5µg total RNA peak on the densitometer trace so this was omitted from Table 23. There appeared to be a stronger hybridization of the chicken MLC2 cDNA probe to the total RNA of the cimaterol samples than the controls. This was apparent at the higher loadings of total RNA in both the dot and slot blots. The relationship between the intensity of the hybridization signal and the quanitity of total RNA in each slot did not reach a plateau in Figure 49 as seen in Figure 47. This may have become apparent at higher loadings of total RNA which were not analysed.

It must be remembered that the chicken MLC2 cDNA probe when used on bovine total RNA did not give a strong hybridization signal in comparison to that of α-actin (Figure 22 and 23). This may have affected the hybridization to the total RNA on the dot and slot blots, although there did not appear to be much nonspecific hybridization in the Northern blot probed with chicken MLC2 shown in Figure 45.

Figure 48: Slot blot of total RNA from cimaterol treated and control bovine L.dorsi probed with MLC2 cDNA. The controls of MLC2 and α-actin cDNA, as well as the quantity of total RNA in each slot, are marked. The cimaterol samples are indicated by X. Hybridization and washing details are described in the text.



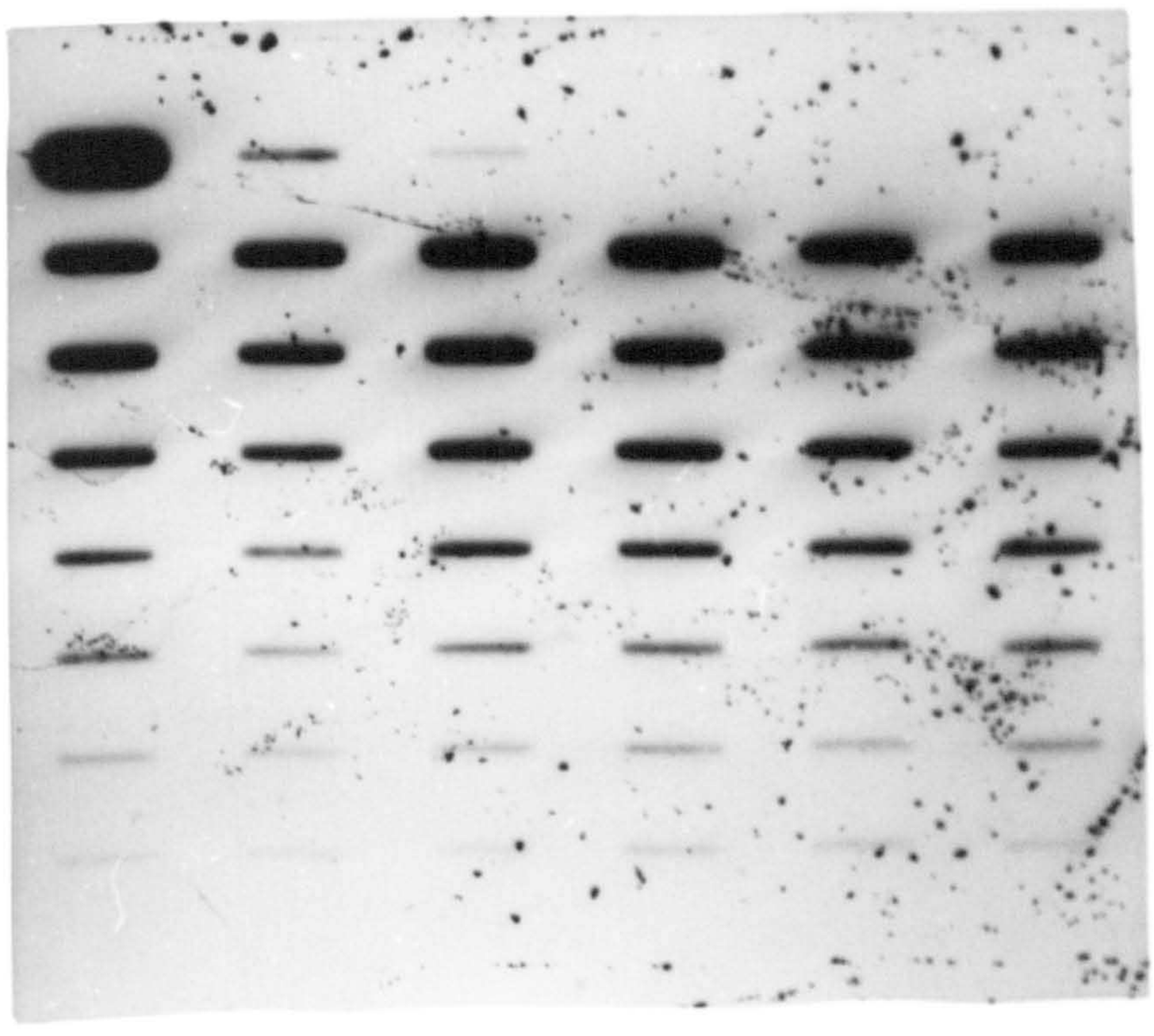


Figure 48: Slot blot of total RNA from cimaterol treated and control bovine L.dorsi probed with MLC2 cDNA. The controls of MLC2 and α-actin cDNA, as well as the quantity of total RNA in each slot, are marked. The cimaterol samples are indicated by X. Hybridization and washing details are described in the text.

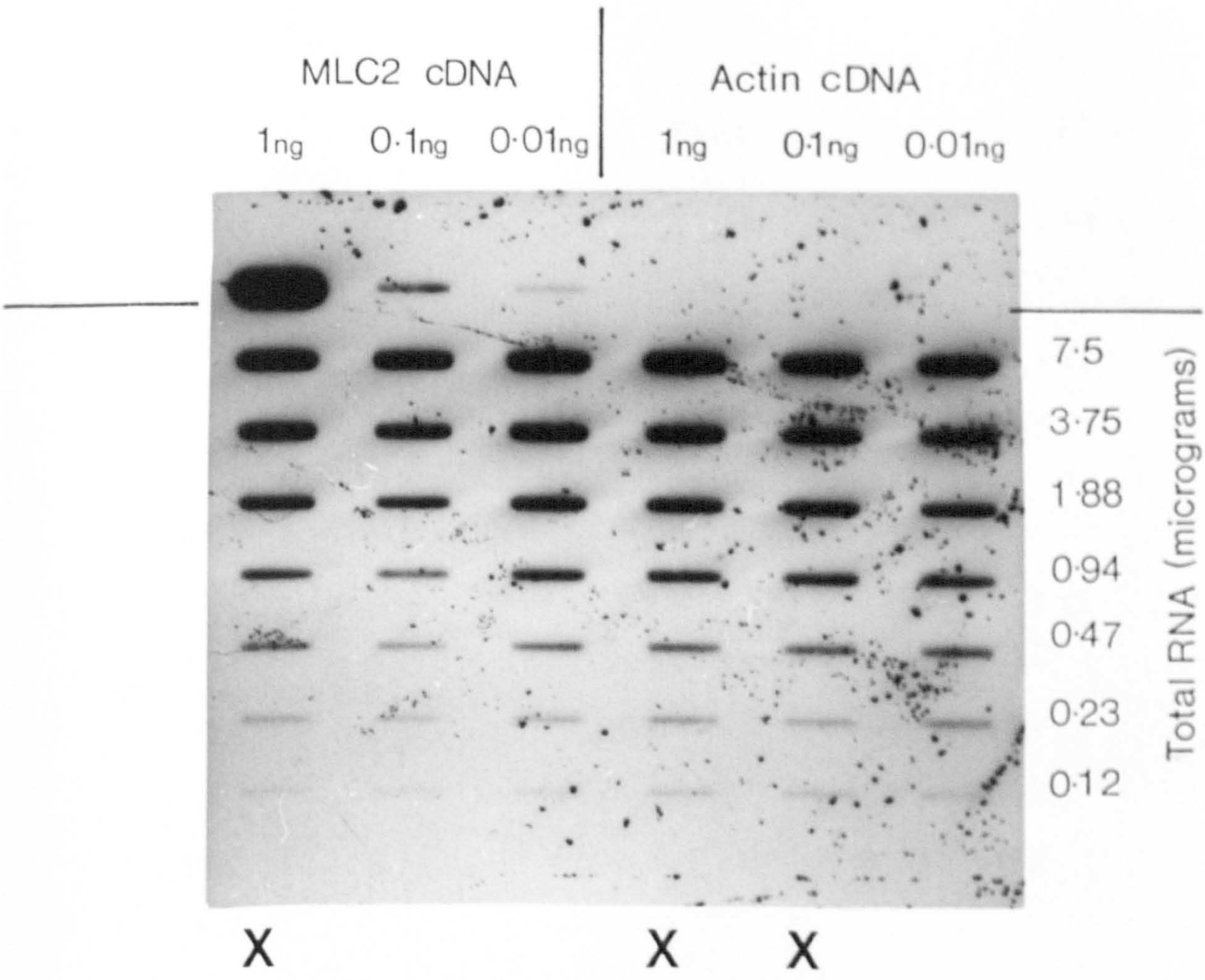


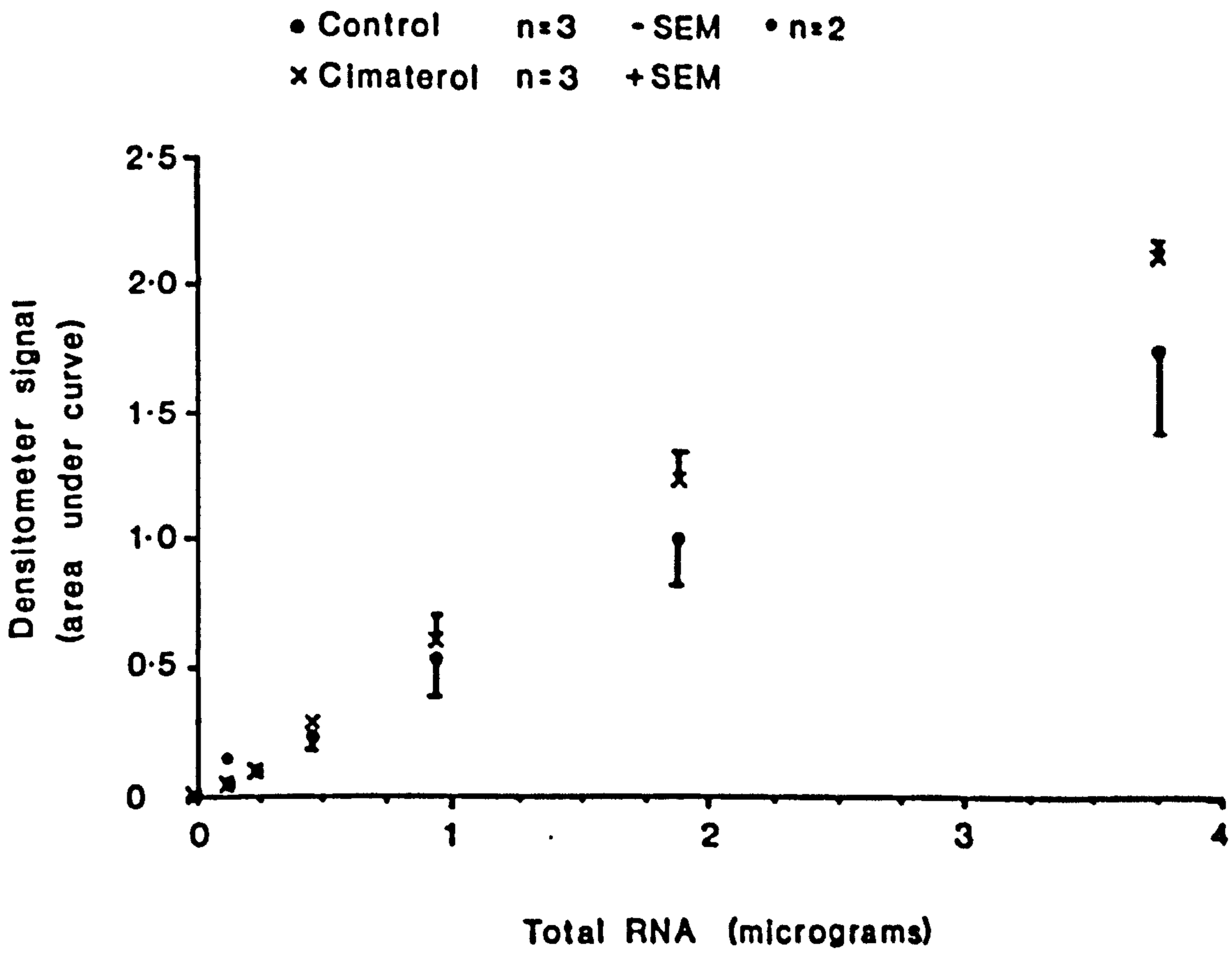
Table 23. Area under the absorbance peaks of the densitometer traces from the chicken MLC2 cDNA probed slot blot (Figure 48).

Dilution	RNA (µg)	Densitometer signal (Area under curve, Arbitrary units) Control (n=3)	Cimaterol (n=3)
1	7.50	-	-
1/2	3.75	1.736 ± 0.304	2.116 ± 0.054
1/4	1.88	1.005 ± 0.189	1.232 ± 0.111
1/8	0.94	0.526 ± 0.147	0.595 ± 0.104
1/16	0.47	0.226 ± 0.053	0.273 ± 0.021
1/32	0.23	0.095 ± 0.022	0.091 ± 0.014
1/64	0.12	0.039 *	0.039 ± 0.010

* n=2

(all values are the mean ± SEM)

Figure 49: The relationship between the total RNA present in each slot and the intensity of the hybridization signal for the MLC2 cDNA probed slot blot (Figure 48) of cimaterol treated and control groups. The data is from Table 23.



Using the slot blotting technique the extracted total RNA was examined for changes in the α-actin mRNA. The mouse α-actin cDNA used for the analysis had been shown to hybridize at a higher stringency than the chicken MLC2 cDNA, 0.1xSSC plus 0.1% SDS at 65°C compared with 2xSSC plus 0.1% SDS at the same temperature respectively (Figure 43 and 45), so would probably be more effective in this type of mRNA quantification. The higher stringency washing would reduce the possibility of nonspecific hybridization.

Serial half dilutions of five samples from each group were made from 7.5µg of total RNA. Three samples of each group were loaded onto one slot blot membrane, whilst the remaining two samples from each group were loaded onto another. The filters were prehybridized in the same volume and had the equivalent quantity of the nick translated labelled mouse α-actin cDNA probe loaded into the hybridization solution. The hybridization was carried out at 55°C, the filters were washed to the same stringency of 0.1xSSC plus 0.1% (w/v) SDS at 65°C. The autoradiograph was scanned as previously described and the results of the integration are shown in Table 24. The data was used to plot the graph in Figure 50.

The same type of curve was produced as in the slot and dot blots probed with chicken MLC2 cDNA. The linear part of the curve appeared to be from 0 to 2µg of total RNA. As with the MLC2 data there appeared to be more α-actin mRNA in the cimaterol samples than the controls especially at the higher loadings of total RNA but on the linear part of the curve there was no apparent difference.

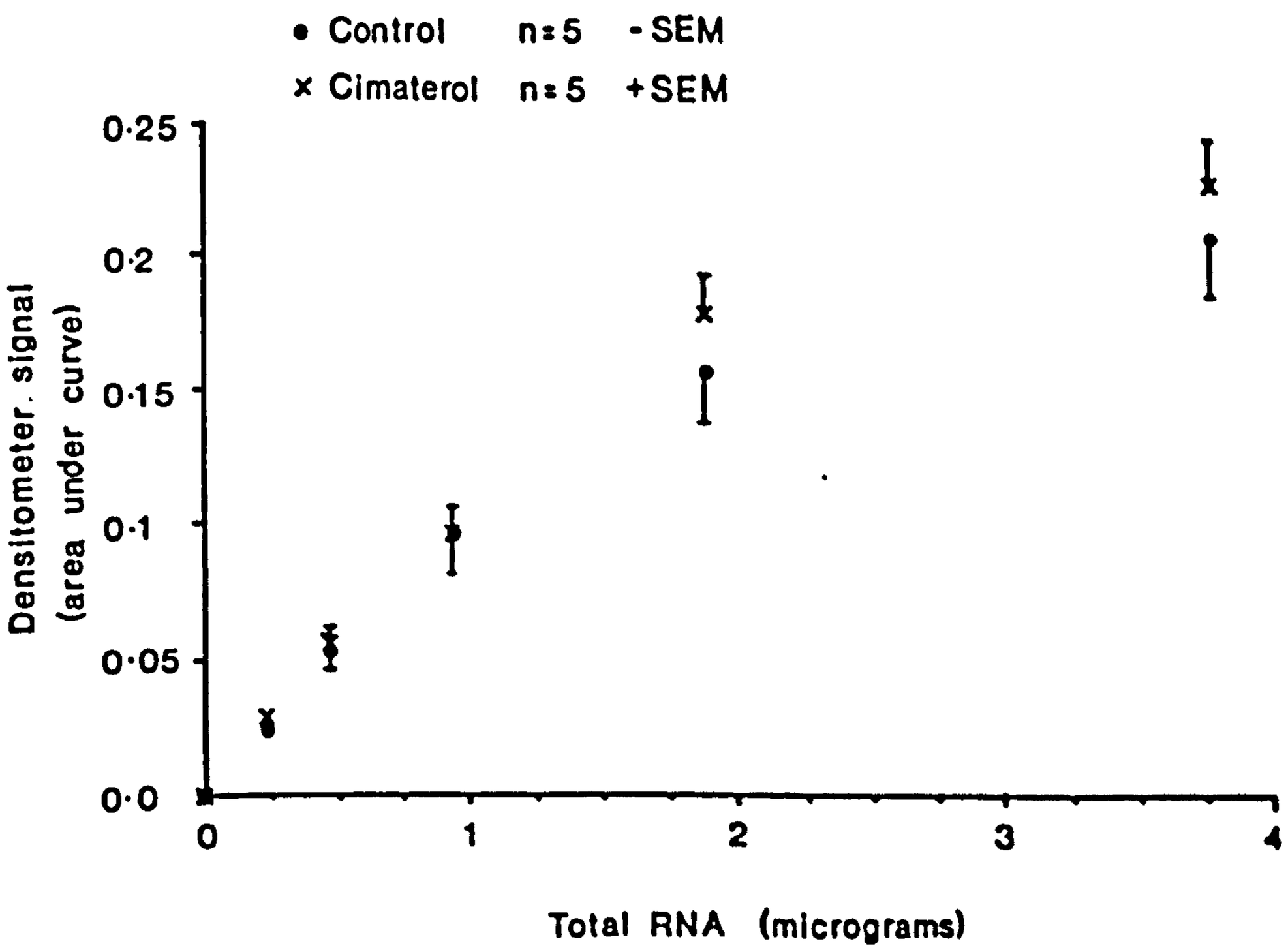
From the slot and dot blots there was no apparent large shifts in the number of mRNA transcripts of two skeletal muscle proteins, MLC2 and α-actin. From this data and the qualitative examination of the in vitro translation protein products generated from cimaterol treated and control bovine L.dorsi total RNA (section 4.2.3.) it appeared that the increased protein accretion in β-adrenergic agonist treatment was probably not due to an large increase in these muscle protein mRNA transcripts.

Table 24. The effect of cimaterol on the hybridization signal produced when probing the slot blots of total RNA from treated and nontreated bovine L.dorsi muscle samples with the mouse α-actin cDNA.

Dilution	RNA (μg)	Densitometer signal (Area under curve, Arbitrary units) Control (n=5)	Cimaterol (n=5)
1	7.50	-	-
1/2	3.75	0.206 ± 0.022	0.227 ± 0.017
1/4	1.88	0.156 ± 0.018	0.179 ± 0.015
1/8	0.94	0.095 ± 0.014	0.095 ± 0.010
1/16	0.47	0.051 ± 0.005	0.051 ± 0.006
1/32	0.23	0.023 ± 0.004	0.028 ± 0.002
1/64	0.12	-	-

(values are the mean ± SEM)

Figure 50: The effect of cimaterol on the relationship between the total RNA present in each slot and the intensity of the hybridization signal for the slot blots probed with mouse α-actin cDNA. The data is from Table 24.



4.2.5. Assessment of Changes in the mRNA Transcripts for the Calpain Large Subunit Isoforms.

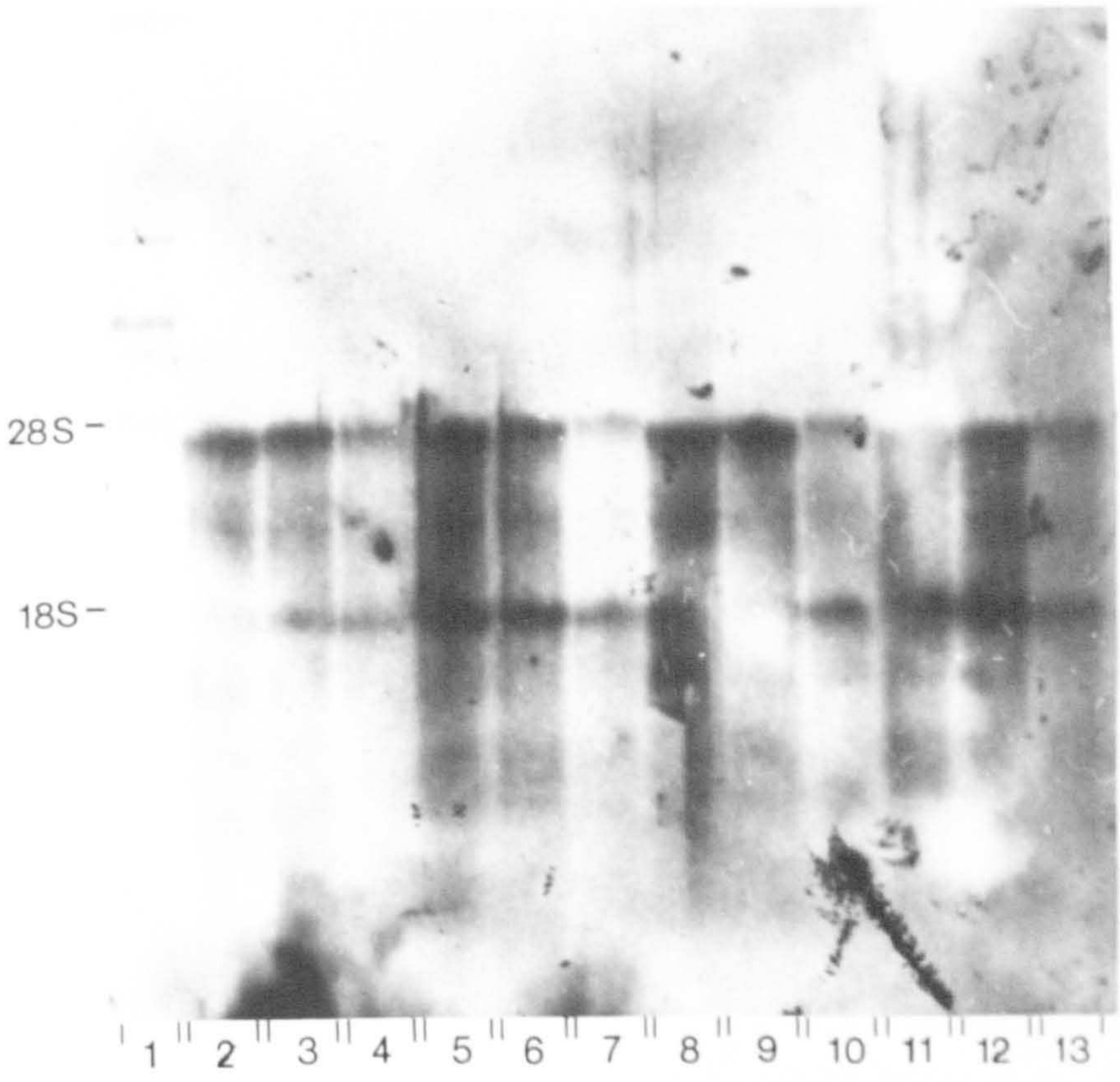
The cDNA inserts from plasmids p42 and p21-16 for human calpain I and II large subunits respectively were used to try and determine any changes in their corresponding mRNA in the bovine L.dorsi total RNA samples.

From earlier work it had been found that to visualize the calpain large subunit mRNA on autoradiographs the optimum quantity of total RNA required on Northern blots was 50µg (section 4.1.4.). To confirm this for the bovine samples 10, 30 and 50µg of total RNA from two samples of each group were electrophoresed on a formaldehyde denaturing gel then Northern blotted (section 3.2.7.(ii) and 3.2.8.). The resulting blot was probed with human calpain I and then calpain II large subunit cDNAs.

The Northern blot was first probed with human calpain I large subunit cDNA labelled by nick translation, hybridized at 55°C and washed to a stringency of 6xSSC at 60°C. There was no clear hybridization signal and the high background on the filter persisted even after washing at higher stringency (Figure 51). Probing of other Northerns of the bovine total RNA samples with the calpain I large subunit gave a similar result as was seen when the human calpain I large subunit cDNA was first characterised (section 4.1.4.). The 1.5kb signal seen in the poly(A)+ enriched RNA (Figure 30) was not visible in this experiment. It may have been obscured by the high nonspecific background. Due to this lack of success in using this probe it was not persisted with.

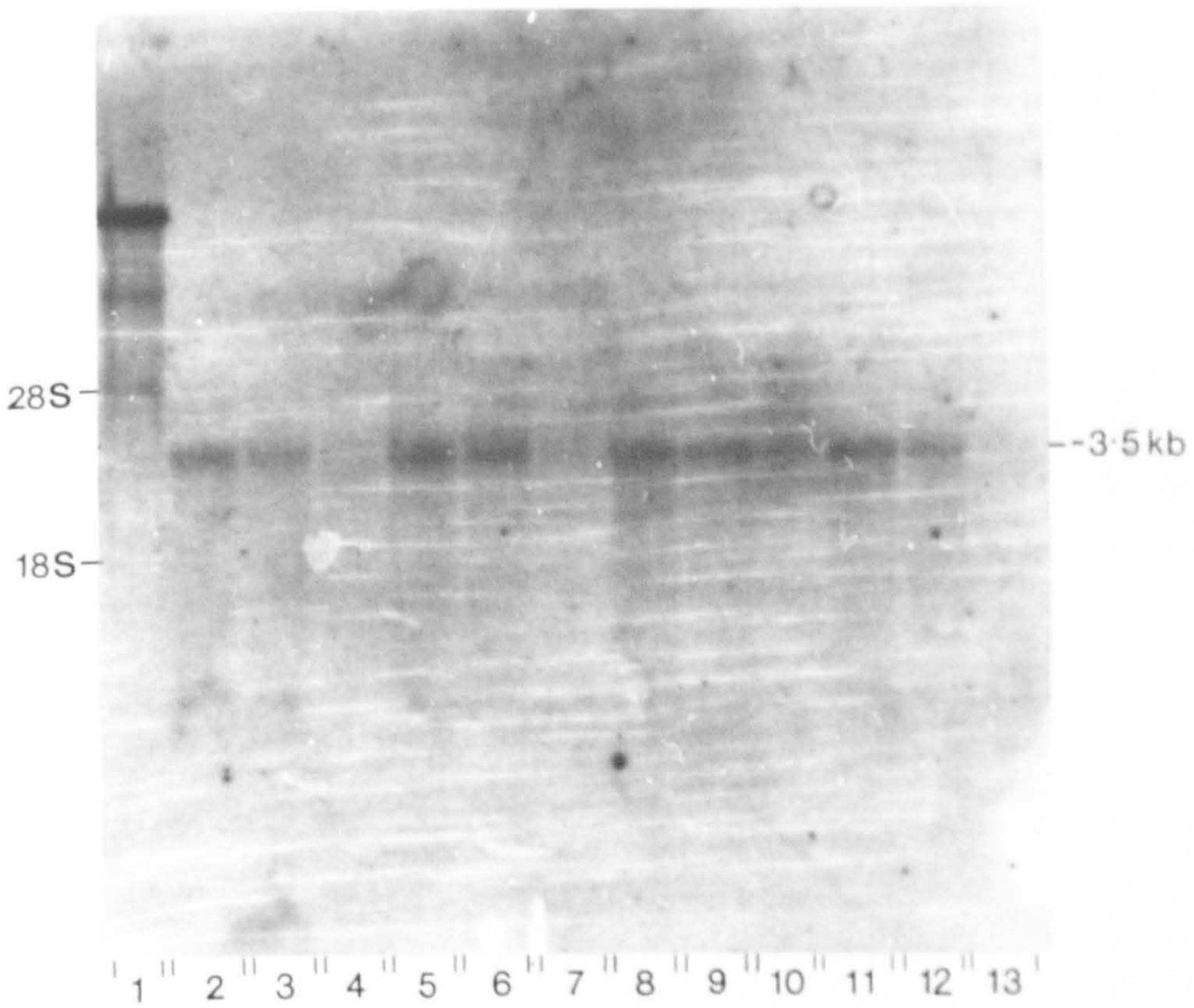
The Northern was reprobed at 55°C with the human calpain II large subunit cDNA labelled by nick translation. The blot was washed to a stringency of 6xSSC at 60°C. A clear hybridization signal could be seen to a mRNA species (Figure 52) as was shown in section 4.1.4. The autoradiograph was scanned using the Ultroscan XL™ Laser Densitometer. Assessment of the area under the calpain II large subunit cDNA hybridization peak was made by integration using a manually selected baseline (section 3.4.14.) (Table 25).

Figure 51: A Northern blot of a range of quantities of total RNA from cimaterol treated and control bovine L.dorsi probed with human calpain I large subunit cDNA. Details are in the text. The ribosomal RNA 'markers', 28S and 18S, are indicated.



Lane No.	Sample
1	1.5µg Hind III lambda DNA markers.
2 to 4	50, 30 and 10µg total RNA, cimaterol treated.
5 to 7	50, 30 and 10µg total RNA, control.
8 to 10	50, 30 and 10µg total RNA, cimaterol treated.
11 to 13	50, 30 and 10µg total RNA, control.

Figure 52: The Northern blot of Figure 51 reprobed with the human calpain II large subunit cDNA. Details are in the text. The position of the 28S and 18S ribosomal RNAs and the size of the hybridization signal for calpain II large subunit are indicated.



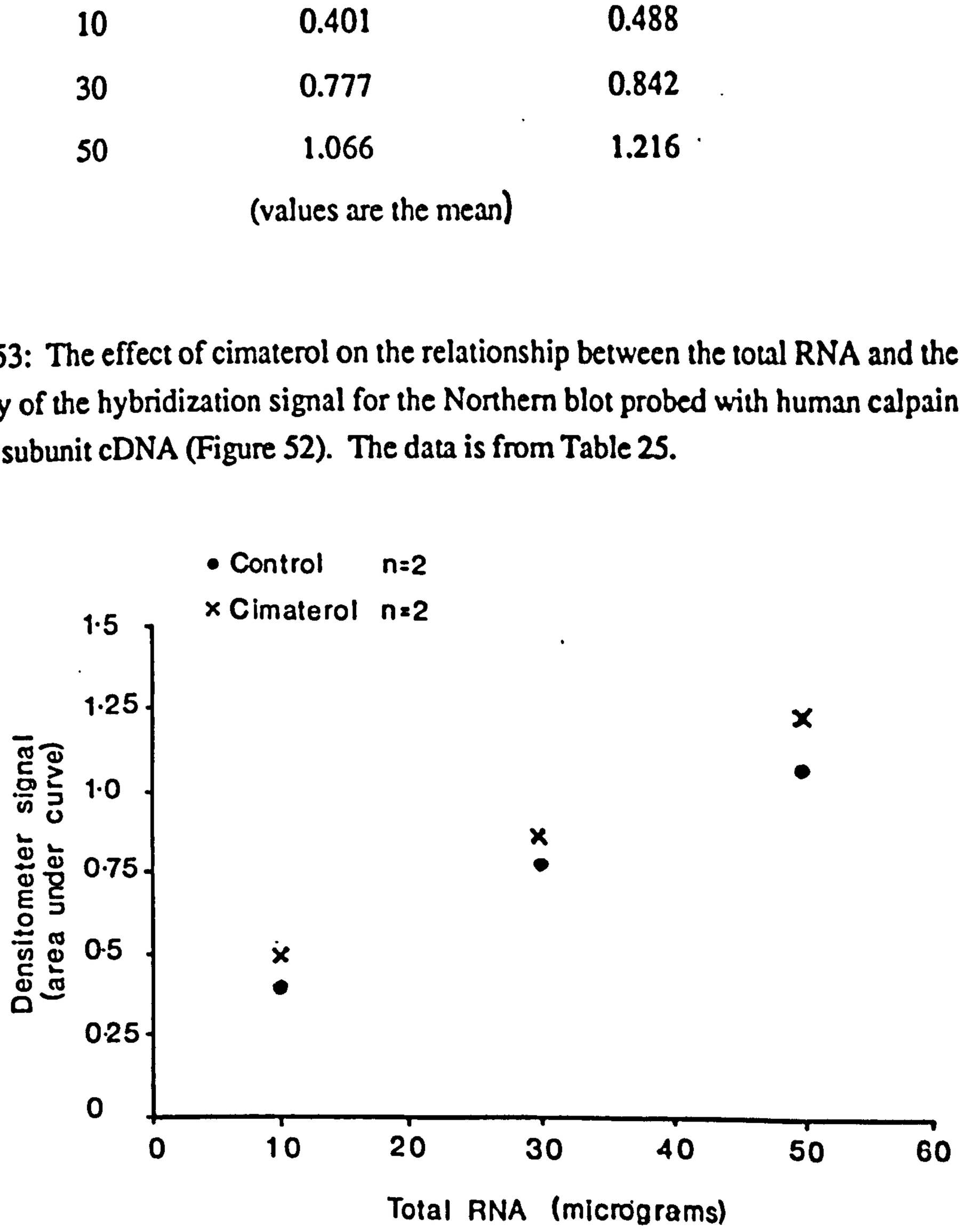
Lane No.	Sample
1	1.5µg Hind III lambda DNA markers.
2 to 4	50, 30 and 10µg total RNA, cimaterol treated.
5 to 7	50, 30 and 10µg total RNA, control.
8 to 10	50, 30 and 10µg total RNA, cimaterol treated
11 to 13	50, 30 and 10µg total RNA, control.

Table 25. The effect of cimaterol on the intensity of the hybridization signal for bovine L.dorsi total RNA probed with human calpain II large subunit cDNA. The Northern blot (Figure 52) was assessed by scanning densitometry.

	Densitometer signal (Area under curve, Arbitrary units))	
RNA	Control	Cimaterol
(μg)	(n=2)	(n=2)
10	0.401	0.488
30	0.777	0.842
50	1.066	1.216

(values are the mean)

Figure 53: The effect of cimaterol on the relationship between the total RNA and the intensity of the hybridization signal for the Northern blot probed with human calpain II large subunit cDNA (Figure 52). The data is from Table 25.



Although there was high background on the autoradiograph the hybridization signal for the calpain II large subunit cDNA was linear with respect to the quantity of RNA loaded (Figure 53). The intensity of the hybridization signal in the cimaterol samples appeared greater than in the controls but it was not significantly so.

A further Northern blot using 50µg of total RNA from the trial samples was made and probed with the human calpain II large subunit cDNA at 55°C. The probe was labelled using a random primer labelling kit (Boehringer) instead of nick translation (section 3.4.9.). This gave a probe of higher specific activity. Activity of labelled cDNAs by nick translation are typically $5x10^8$ dpm/µg whilst random primer labelling gives probes of $5x10^9$ dpm/µg, when using the procedures suggested by the manufacturers of the kits (Amersham). Because of the low copy number of calpain II large subunit mRNA, higher specific activity of the probe was advantageous. Figure 54 shows the Northern probed with the calpain II large subunit cDNA and washed to a stringency of 2xSSC at 65°C, after hybridization at 55°C.

In order to obtain a clear signal of hybridization, the membranes probed with the calpain II large subunit cDNA had to be autoradiographed for 5 days, due to the low copy number of the calpain mRNA transcripts. As a result there was high background on the autoradiograph. This was compounded by the lower stringency of washing than was required for the actin and MLC2 cDNA probes.

The autoradiograph was scanned. The traces produced are shown in Figure 55. The intensity of the hybridization signal was quantified by integration of the peak area, Table 26.

Although there was a higher value for the cimaterol samples to that of controls the difference was not significant. Probing Northerns of total RNA from treated and control L.dorsi with the human calpain II large subunit cDNA in similar experiments to the one described above gave similar results, although they tended to have higher nonspecific background. There was an increase in the intensity of the signal for calpain II large subunit mRNA in the treated samples but this was never significant.

Figure 54: Human calpain II large subunit cDNA probed Northern blot of total RNA (50µg) from cimaterol treated and control bovine L.dorsi. Hind III lambda DNA markers (1.5µg) are indicated by the letter M and the cimaterol samples are marked with 'X'. Conditions of hybridization and subsequent washing are outlined in the text. The position of the 28S and 18S ribosomal RNAs are shown and the size of the hybridization signal for calpain II large subunit.

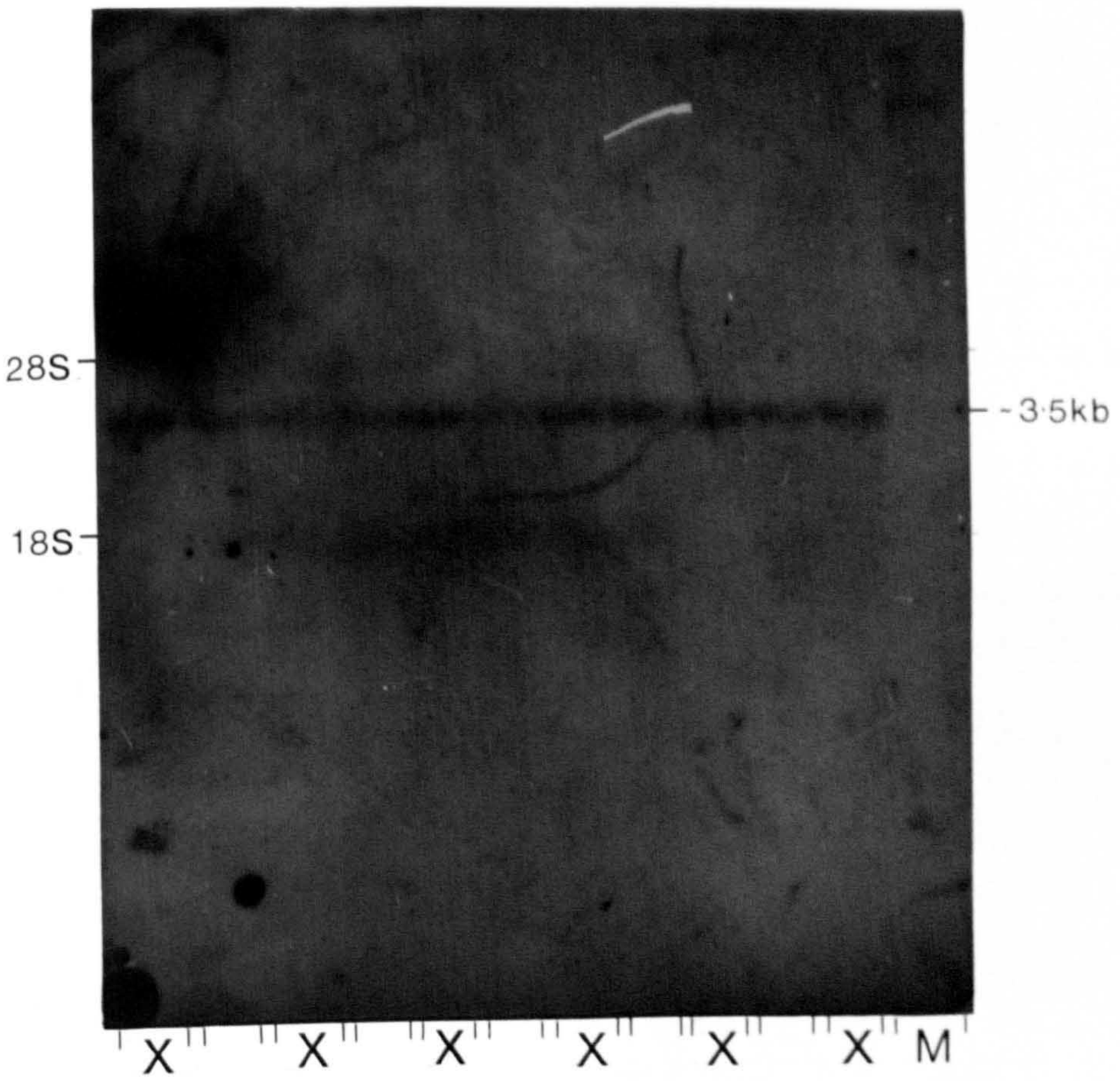


Figure 55: Traces of the densitometer scanned human calpain II large subunit cDNA probed Northern blot (Figure 54).

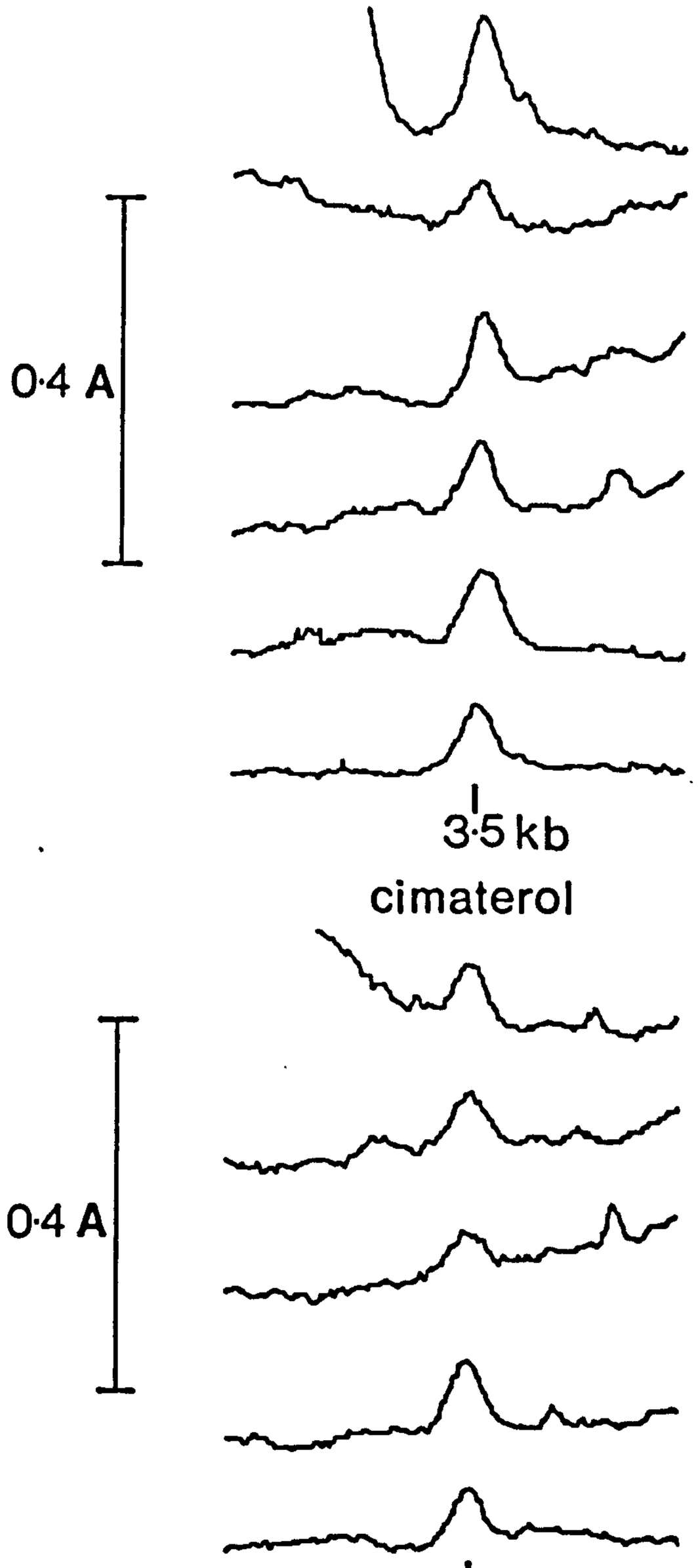


Table 26. The effect of cimaterol on the intensity of the hybridization signal for the human calpain II large subunit cDNA probed Northern blot of Figure 54.

	Control (n=5)	Cimaterol (n=6)	SED	P
Area by Integration (Arbitrary units)	0.234 ± 0.023	0.313 ± 0.032	0.041	> 0.05

(values are the mean ± SEM)

Attempts were made to quantify the changes in the expression of calpain II large subunit between the samples by slot blotting. This was not possible as not more than 15µg of skeletal muscle total RNA samples could be loaded successfully onto the Hybond-N membrane via the slot blot apparatus. Northern blots had to be used as a means of quantifying changes in the calpain mRNA in total RNA samples as over 30µg of total RNA was required to give a clear hybridization signal, as seen on Figure 52.

4.2.6. Changes in the Expression of Calpastatin mRNA.

The PCR generated bovine calpastatin cDNA was used to probe Northern blot of 50µg of L.dorsi total RNA samples from the bovine β-agonist trial. The calpastatin PCR cDNA insert mixture, from pG3p13 and pG3p21, was labelled using the random primer method (section 3.4.9.). Hybridization was at 55°C and the Northern was washed to a stringency of 2xSSC plus (w/v) 0.1% SDS at 65°C. In order to obtain a clear autoradiograph of the hybridized calpastatin PCR cDNA the probed Northern was left for 7 days to develop.

As with the probing of the bovine poly(A)+ RNA Northern blot (section 4.1.5.) there were three hybridization bands at approximately 5.1-5.0, 3.8-3.7 and 2.9-2.6kb (Figure 56). The 2.9-2.6kb hybridization band was more distinct than in the poly(A)+ enriched RNA Northern blot (Figure 36). There was a clearly visible difference in the strength of the signal at 3.8-3.7kb and to a lesser degree at 5.1-5.0kb. This was not due to a discrepancy in the loading of the total RNA samples as this filter was reprobed with the human calpain II large subunit cDNA probe and there was no significant difference in the hybridization signal between the samples (Figure 54).

Figure 56: Bovine calpastatin PCR cDNA probed Northern blot of total RNA (50µg) from the cimaterol treated and control bovine L.dorsi. Hind III lambda DNA markers (1.5µg) are indicated by the letter M and the cimaterol samples are marked with 'X'. Conditions of hybridization and washing are outlined in the text. The position of the 28S and 18S ribosomal RNAs are shown along with the size of the hybridization signals for calpastatin.

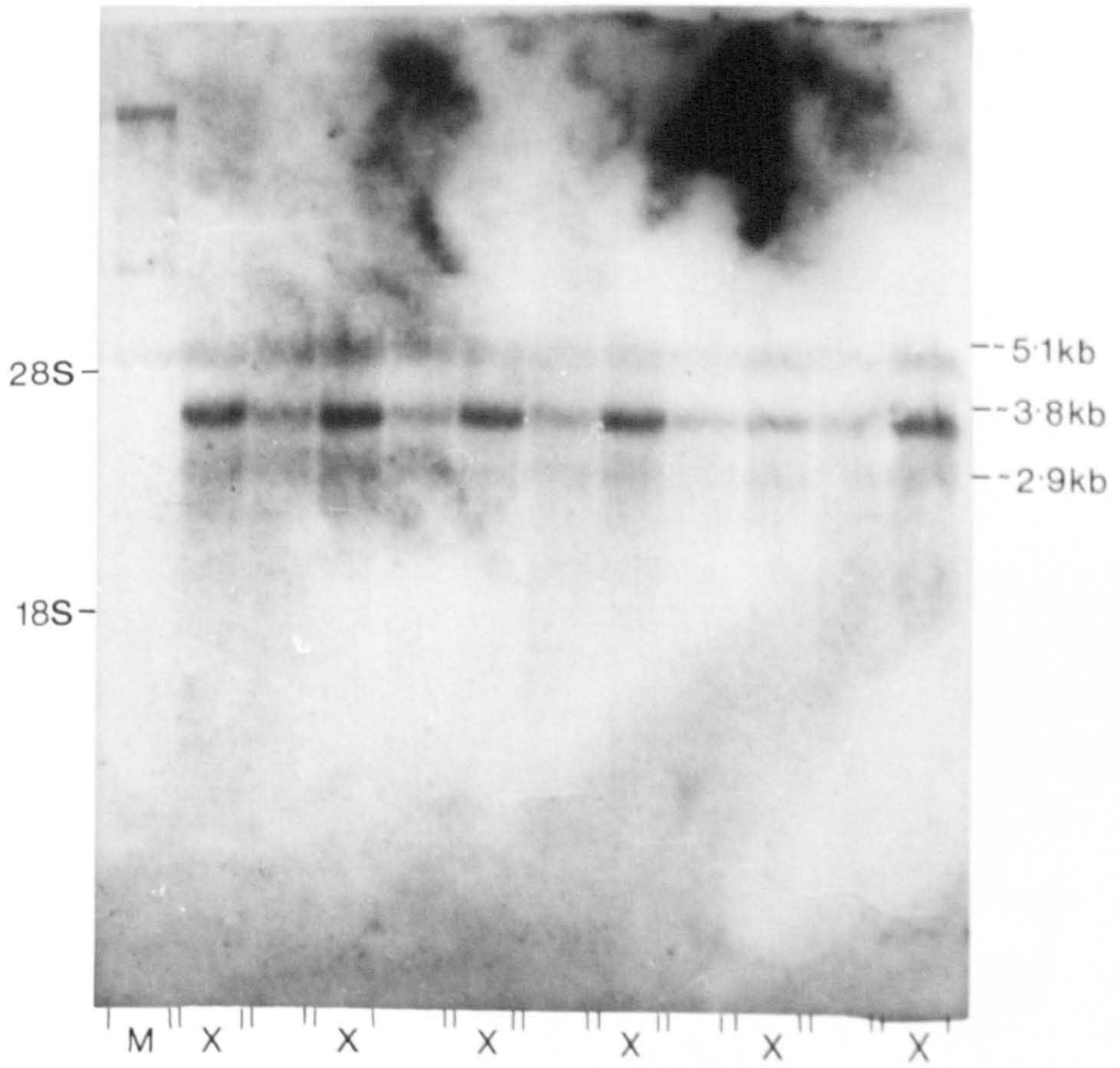
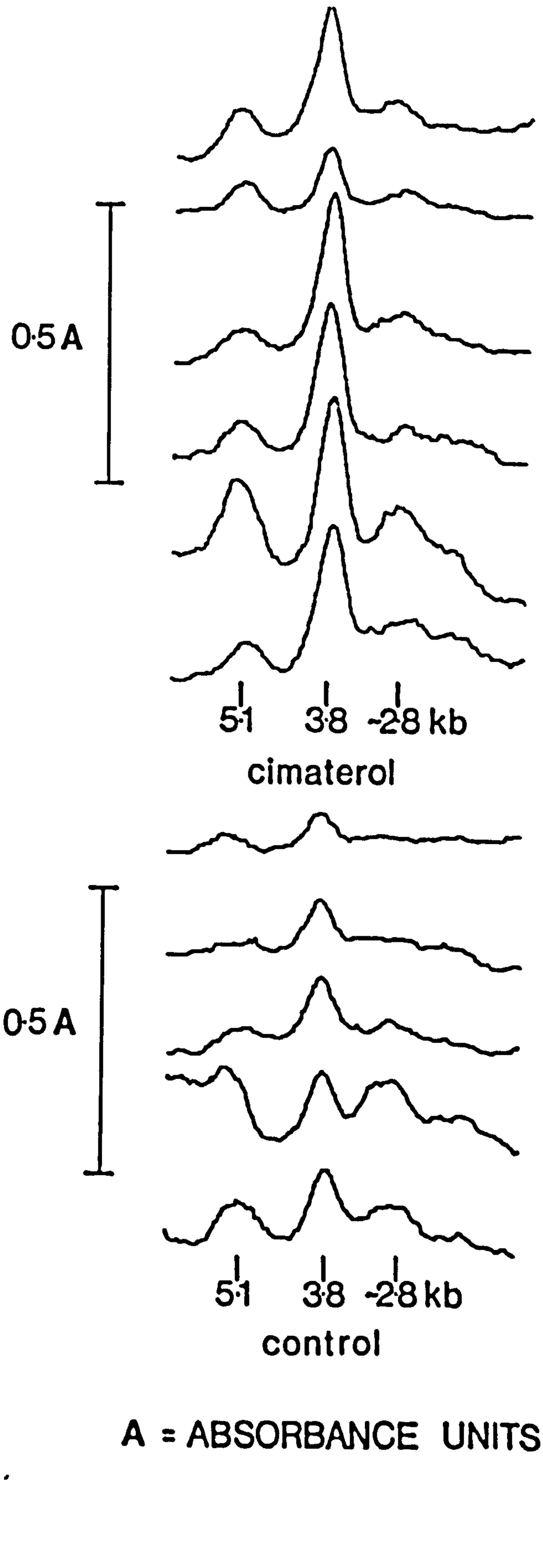


Figure 57: Traces of the densitometer scanned bovine calpastatin PCR cDNA probed Northern blot shown in Figure 56.



The autoradiograph was scanned and the resulting traces are shown in Figure 57. The difference between the cimaterol and control samples was clear. Areas under the two definite peaks at 5.1-5.0 and 3.8-3.7kb were integrated along with the area of the speculative peak in the 2.9-2.6kb region (Table 27 and Figure 27).

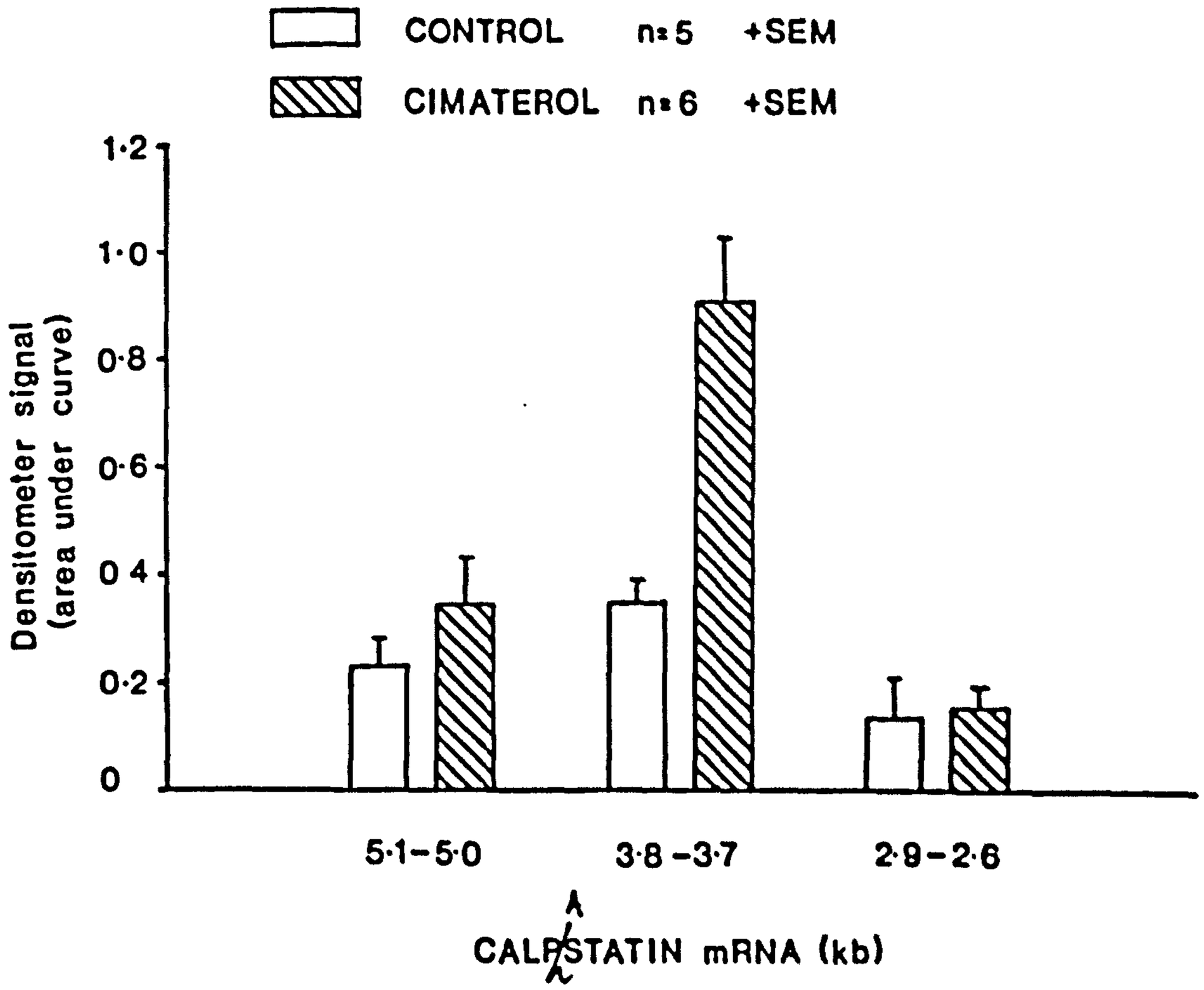
Although for all three hybridization bands the cimaterol samples were of greater intensity, this was only significant with the signal at 3.8 kb.

Table 27. The effect of cimaterol on the intensity of the three hybridization signals for the bovine calpastatin PCR cDNA probed Northern blot of Figure 56.

RNA Size	Densitometer signal (Area under curve, Arbitrary units) Control (n=5)	Cimaterol (n=6)	SED	P
5.1-5.0kb	0.230 ± 0.054	0.346 ± 0.085	0.105	> 0.05
3.8-3.7kb	0.351 ± 0.042	0.915 ± 0.118	0.135	< 0.05
2.9-2.6kb	0.139 ± 0.073	0.157 ± 0.043	0.810	> 0.05

(values are the mean ± SEM)

Figure 58: The intensity of the hybridization signals for calpastatin mRNA in cimaterol treated and control bovine L.dorsi total RNA samples.



Chapter 5. Discussion.

5.0. Introduction.

Previous observations from within our laboratories have shown that there is an effect on the activities of the calpain system components (calpain I and II and calpastatin) associated with β-agonist induced skeletal muscle hypertrophy. The work carried out in this thesis was undertaken to further examine this effect by moving the attention toward the possible means by which the changes in activity could be brought about. Therefore this thesis concentrated on whether the changes in activity could be brought about by alterations in the mRNA expression of the calpain system components. During the course of these experiments observations were also made on the effects of the β-agonist cimaterol on the expression of mRNA species for myofibrillar proteins α-actin and MLC2 along with the translational capabilities of the isolated total RNA.

These observations are considered in the following sections and are related to previous studies which suggested that augmented protein accretion was brought about by decreases in protein degradation (115-118). Particular attention is focused on the role of the changes in calpastatin mRNA expression and its probable subsequent effect on the inhibition of the calpain proteolytic system and the relationship of this to the decline in protein breakdown.

However the first sections of this discussion deal with the observations made on preparing total RNA from skeletal muscle and the use of different types of nucleic acid hybridization probes for calpain. This is followed by a detailed examination of the bovine calpastatin cDNA generated by PCR, how it relates to the calpastatin cDNAs already isolated and the implications of the mRNA hybridization signals seen when the PCR cDNA was used for Northern analysis of bovine total and poly(A)+ enriched RNA.

5.1. Isolation of Total RNA and the Use of Hybridization Probes to Calpain mRNA.

Extraction of total RNA.

The development of techniques for the isolation of intact total RNA from skeletal muscle were a result of ongoing work carried out on the detection of various mRNA species. As shown in the Figures of section 4.0.1. the quality of the total RNA improved during the experiments carried out to compare extraction methods and assess various cleaning procedures. The main objectives of total RNA isolation was the physical removal of protein by denaturing with phenol/chloroform then the adoption of effective reprecipitation techniques which removed DNA from the samples. Although the method may not have been absolutely quantitative it did give reproducible results which allowed comparison of extractable intact total RNA in the bovine trial (section 4.2.2.).

The improvement in the quality of the RNA extracted was essential for the ability to produce proteins from mRNA by <u>in vitro</u> translation (section 4.0.3.). This was also achieved by the modification of the <u>in vitro</u> translation 'cocktail' to give an environment where the rabbit reticulocyte lysate was effective on skeletal muscle mRNA.

Oligonucleotide probes for calpain large subunit.

The mRNA for the calpain isoforms and calpastatin are expressed at very low levels in most tissues(159,199), approximately 1 picogram of chicken skeletal muscle calpain large subunit per microgram of total RNA, so they are difficult to detect. When the oligonucleotide I was used as a probe on poly(A)+ RNA prepared from chicken skeletal muscle a clear hybridization signal of the expected size for the calpain large subunit mRNA at 3.5kb (14) was detected (section 4.1.1.). This hybridization took place at relatively high stringency. Although the oligonucleotide I probe had been chosen to have high homology across species and was selected from domain III, which was believed to have no homology to non-calpain cDNA sequences, it was difficult to produce a specific hybridization signal to calpain large subunit mRNA across species even when it was used in low stringency conditions (section 4.1.1.). These hybridization conditions were chosen to encourage hybrids to form to related sequences

across species, as the melting temperature (T_m) of the hybrid would be lower than that of a 100% matching sequence.

The failure of the oligonucleotide I probe was very likely due to mismatches between its sequence and the appropriate region in bovine calpain large subunit mRNA which would seriously effect hybridization capability of such a short probe, only 39 nucleotides. Although reducing stringency enhanced the possibility of detecting calpain large subunit mRNA across species the likelihood of hybridization to similar unrelated sequences increased. The chicken calpain large subunit is believed to be a calpain II-like isoform (14) so the oligonucleotide I probe was expected to hybridize to bovine calpain II large subunit mRNA. However, the oligonucleotide I probe tended to hybridize nonspecifically, particularly to the rRNA, so that the high background masked any calpain mRNA bands (Figure 21). There was faint band at approximately the size expected for calpain large subunit but the two isoforms are reported to be near the same length so it would in any case be impossible to decide which large subunit isoform had been detected (15,16,17).

Griffiths et al (196) successfully used end labelled oligonucleotides (33mers) to detect the mRNA encoding closely related proteins. They examined the mRNA expression of G-protein subunits in streptozotocin induced diabetes in rats. Although the mRNAs they detected were closely related they were able to produce specific hybridization without nonspecific background on Northern blots of total RNA. Their oligonucleotide probes were selected from the cDNA of the species in which they were examining the changes in mRNA. They observed that a 30% mismatch between oligonucleotides was sufficient to prevent cross-hybridization. For chicken large subunit oligonucleotide I probe the percentage mismatch to other species was between 15 and 21% (Table 8). This may have been sufficient mismatch to prevent hybridization in cattle but could be complicated by the distribution of the mismatches, closely grouped mismatches strongly affecting hybridization.

The problems of nonspecific hybridization may have been the result of overloading the hybridization solutions with labelled probe. However the quantities used

were within the guide-lines recommended by textbooks and labelling system manufacturers (167,175,195). Reprobing the Northern blots with mouse α-actin and chicken myosin light chain 2 cDNAs gave no acute nonspecific hybridization, even though they were not specific to the bovine total RNA probed and were used in higher concentrations than the oligonucleotide I probe, see Figure 22 and 23. This was because of the length of the cDNAs used, the proteins being conserved between species and the relatively high expression of their mRNA in skeletal muscle.

In order to try to detect the mRNA for calpain in total RNA samples and across species using oligonucleotides a different labelling technique was employed. This was attempted after PCR generation of a cDNA had failed for the reasons outlined in section 4.1.2.. The technique of specific primer extension clearly gave a oligonucleotide probe of higher specific activity (section 4.1.3.). This would make it more effective in the detection of low copy number calpain mRNA. But as with the end-labelled oligonucleotide it was unsuccessful in producing specific hybridization to the mRNA in chicken skeletal muscle total RNA in low stringency conditions. By development of this technique a better oligonucleotide probe possibly could have been produced but the effects of nonspecific hybridization would probably still have been a problem in low stringency conditions due to the probe binding unrelated sequences. Production and refinement of bovine poly(A)+ RNA may have improved hybridization signals, but insufficient tissue was available in the trial.

The human calpain I and II large subunit cDNAs

Even given clear hybridization signals it would have been difficult to distinguish between the two isoforms for calpain large subunits using oligonucleotide probes derived from the chicken skeletal muscle isoform because of lack of high homology specifically toward either calpain I or calpain II. By using the human calpain I and II large subunits cDNAs (16,17) it was expected that a greater degree of specificity could be achieved. The human calpain I large subunit cDNA failed to detect convincingly the expected 3.5kb signal in bovine skeletal muscle total and poly(A)+ enriched RNA Northern blots (Figure 29 and 30). The possible reasons for this were discussed in section 4.1.4.. The human

calpain I and II large subunit cDNAs were subjected to restriction endonuclease analysis (appendix A) before use and results agreed with the data of Aoki et al (16) and Imajoh et al (17) respectively. Full characterization could only have been achieved by sequencing, but insufficient time was available for this.

As a result of nonspecific hybridization the use of calpain I large subunit cDNA was abandoned for the bovine trial. The calpain II large subunit cDNA weakly hybridized to a mRNA species in bovine skeletal muscle total RNA (Figure 28 and 30) which was in agreement with the 3.5kb size predicted for the isoform (15,17). A weak signal was probably expected because of its cross-species application and the low copy number of the mRNA. Similar hybridization signals were shown in autoradiographs from the the original publication (17). There was a possibility that the human calpain II large subunit cDNA probe did cross hybridize to bovine calpain I or the skeletal muscle specific putative 'calpain isoform' p94 (28,29) at the low stringency used in the hybridization experiments. However, it had been reported that by using the 3' non-coding region of the full length cDNA the probes were specific to their own isoforms (15). The clarity and sharpness of the hybridization band when using the calpain II large subunit cDNA suggests it was specific to its analogous isoform in bovine skeletal muscle total RNA. Had time permitted, the generation of derivative probes, perhaps in the noncoding region may have given enhanced signals for the calpain I large subunit (see section 4.1.4)

5.2. The Bovine Skeletal Muscle Calpastatin mRNA PCR cDNA Probe. Calpastatin PCR cDNA sequence analysis.

The cDNA probe to bovine skeletal muscle calpastatin was made from total RNA by PCR using the knowledge gained whilst trying to generate the chicken skeletal muscle calpain large subunit cDNA using PCR (section 4.1.2.) and the calpastatin cDNA sequence information which had been published in the literature (52-54). From these cDNAs PCR primer oligonucleotide sequences for calpastatin were selected (section 4.1.5.) which subsequently have been used to generate calpastatin PCR cDNAs from rat,

pig and sheep total RNA (work carried out by Dr R.S.Gilmour, AFRC, Institute of Animal Physiology and Genetics Research, Cambridge.).

The cDNA produced was not full length but was predicted to cover domain 4 of calpastatin. The bovine PCR cDNA did align with domain 4 of the calpastatin cDNAs for rabbit (52), pig (53) and human (54). There is high homology across the four primary sequences (66-75%) particularly in the two α-helical regions and inhibitory central consensus sequence (59) (Figure 35, Table 11). However there is an amino acid substitution at a critical position in the central consensus sequence. As outlined in the review of the literature (section 2.2.1.) both groups led by Murachi and Suzuki have shown there is a 'core' central consensus sequence;

Thr-Ile-Pro-Pro-(X)-Tyr-Arg

which appears to be critical for inhibitory activity (56,60,61). In the amino acid sequence predicted from the bovine PCR cDNA (Figure 35) the above appeared as;

Thr-Ile-Pro-Pro-Lys-Tyr-Gln

The lysine residue is in a non-conserved position X but in domain 4 of the other primary sequences this is glutamic acid (Figure 59). The substitution of glutamine for arginine is in a position which is highly conserved. Arginine residues are present in this position across all the domains in all the previously examined species, Figure 59.

In the bovine calpastatin PCR cDNA the glutamine codon is CAA whilst the possible arginine codons are CGT, CGC, CGA, CGG, AGA, AGG. As the position of the glutamine in the central consensus sequence is against the trend seen in the calpastatin sequences so far isolated the codon change could be a result of a PCR induced mutation. However from the two PCR products, pG3p13 and pG3p21, the codon in this position was the same, CAA, in both central consensus sequences. The reported base substitution error for Taq polymerase is one in each 9000 nucleotides polymerized, whilst frameshift errors are one in 41000 (202). These mutations are caused by Taq polymerases inability to proof-read 3' to 5' sequences it has polymerised. A frameshift error is unlikely in the calpastatin PCR cDNA as the predicted primary sequence in this region is virtually

Figure 59: Amino acid sequence comparison of the inhibitory central consensus region of calpastatin. Regions shown are those as defined by Uemori et al (53), of all four domains in the pig, rabbit and human calpastatin. The residues not identical to the pig sequence are indicated in bold whilst the bovine amino acid sequence is shown aligned to domain four. Residues conserved or conservatively substituted across all the domains in the pig, rabbit and human sequences are indicated with an asterisk.

DOMAIN 1	pig	195 L G K R E V T L P P K Y R 207
	rabbit	194 L G K R E V T I P P K Y R 206
	human	172 L G K R E V T I P P K Y R 184
DOMAIN 2	pig	331 C G E D D E T V P P E Y R 343
	rabbit	331 C G E D D E T V P Δ E Y R 343
	human	293 C G E D D E T I P S E Y R 305
DOMAIN 3	pig	471 L G E K E E T I P P D Y R 483
	rabbit	474 L G E K E E T I P P D Y R 486
	human	435 L G E K E E T I P P D Y R 447
DOMAIN 4	pig	607 L G E R D D T I P P E Y R 619
	rabbit	611 L G E R D D T I P P E Y R 623
	human	572 L G E R D D T I P P E Y R 584
		* * * * * * *
	bovine	L G E R D D T I P P K Y Q

completely homologous to other sequences, apart from the two residues lysine and glutamine (Figure 59).

As the two bovine calpastatin PCR cDNA sequences are in agreement at the glutamine codon it is unlikely that the difference of these to the other species is caused by nucleotide substitution. This is further confirmed when this codon nucleotide sequence in domain 4 is examined in other species. The arginine residue is coded for by AGG, AGA and AGA in rabbit, pig and human calpastatin cDNAs respectively. If the presence of glutamine is due to a mutation of an arginine codon the expected original codon would be AGX. Assuming that the nucleotide sequence is conserved across species a double nucleotide substitution would have to take place in order to get a substitution to glutamine. However because of the low copy number of calpastatin mRNA mutations early on in PCR are likely to have greater effect in producing cDNA sequence mutations than amplifications on a more common sequence.

As the arginine residue is conserved across the domains and species of the calpastatin sequences published in the literature (52-54) it may be critical for the inhibitory action of the sequence. The substitution to a glutamine residue is not a conservative one. The glutamine side chain is polar but arginine's is strongly basic being positively charge at neutral pH. In order to confirm that the glutamine residue predicted in the bovine calpastatin is not due to a PCR error the complete cDNA needs to be isolated, or the PCR generation of the cDNA repeated.

The appearance of lysine in the position X of the central consensus sequence is different from the residue seen in domain 4 of the other sequences, glutamic acid. However examination of the other domains shows that this residue is either acidic or basic and it is not conserved across domains (Figure 59).

Although studies have identified the two α-helical and particularly the central consensus region of the calpastatin domains as being involved in calpain inhibition (59), the residues that directly interact with the enzyme have yet to be elucidated. The experimental evidence does point toward the residues in the central consensus region, as peptides from this inhibit calpain (56,60,61). Calpastatin's specificity toward calpain and

the lack of an absolutely specific synthetic calpain inhibitor suggests that it inhibits the enzyme by some other means rather than just direct active site interaction as seen in the general cysteine proteinase inhibitors. However, the homology of calpain's active site to other cysteine proteinases is not high (203). A low level of similarity may be sufficient to allow the synthetic inhibitors to act on cysteine proteinases as well as calpain but the divergence is great enough to allow calpastatin to act specifically. The ability to achieve specific calpain inhibition, perhaps through the use of peptides from the calpastatin sequence, will aid the investigation into the physiological role of the proteinase.

Hybridization of the calpastatin PCR cDNA to multiple mRNA species.

The cDNA was used as a specific probe to bovine skeletal muscle calpastatin mRNA. The cDNA probe hybridized to three mRNA species. Those at 5.1-5.0kb, 3.8-3.7kb were clear bands whilst at 2.9-2.6kb there was a smear in both total and poly(A)+ RNA Northern blots (Figure 36 and 56).

Multiple mRNA signals for calpastatin have been identified in rabbit and human (52,55). The data obtained for the human was from the use of pig calpastatin cDNA on RNA from human haematopoietic cell lines and the autoradiographs produced were unclear (55). The mRNAs in both experiments were 3.8, 3.0 and 2.5kb. The studies did not isolate a 5.1kb mRNA species. The large mRNA was found in Northern blots of bovine total RNA as well as poly(A)+ enriched RNA after high stringency washing (0.1xSSC plus 0.1% SDS at 65°C), which suggests there is specific hybridization of the bovine calpastatin PCR cDNA probe.

Examination of the 3'non-coding region of rabbit calpastatin cDNA showed that there were multiple poly(A) addition sites coded for by the sequence AATAAA. Three of these are located at appropriate positions to account for the multiple mRNAs (52). In the pig sequence there are a couple of alternative poly(A) addition sequences to those at the 3' end of the cDNA, located at approximately 3.2kb of the 4kb cDNA. However these consist of the less common sequence ATTAAA which is found in 10% of poly(A) sites (204). In describing the isolation of the pig calpastatin cDNA, Takano et al (53) did not show any Northern blots of the cDNA used against pig mRNA; without this data it

impossible to state whether the observation of a 5.1kb hybridization product to bovine calpastatin is an isolated occurrence, or pig calpastatin contains multiple mRNAs for calpastatin. The lack of as many poly(A) addition site sequences in the pig 3' noncoding region compared to the rabbit suggests that the positions of these sites and the resulting lengths of the mRNAs is not conserved from species to species. Confirmation of the existence of a 5.1kb bovine calpastatin mRNA awaits the isolation of the cDNA or the generation of cDNA to the 3'noncoding region by PCR.

The multiple calpastatin mRNAs identified in bovine skeletal muscle mRNA could be generated by three possible mechanisms:

1) There could be more than one gene for calpastatin.

2) The mRNAs could be from one gene and are the result of alternative splicing of transcripts.

3) Alternative utilization of multiple poly(A) addition sites in the 3' untranslated region of the single calpastatin gene.

The number of calpastatin genes has yet to be determined. The calpain subunits mRNAs are similarly expressed at low levels and are the product of single genes as far as is presently known (161).

Alternative splicing can produce multiple mRNAs from a single gene (204,205). One of the mechanisms by which this can be achieved is through the use of alternative poly(A) addition sites which may dictate the selection of different exons making up the mRNA (204,205). The resulting translation product is different from each alternatively spliced mRNA. This method of splicing seems to occur when the poly(A) addition sites are located in different exons and it usually results in the tissue-specific expression of only one isoform of the mRNA. Although calpastatin exists as multiple isoforms, liver and erythrocyte sub-types as described in section 2.2.1., the different calpastatins identified are thought to be the result of post-translational modification (30). The cDNA cloning experiments have not detected 5' truncated cDNA isoforms (52-54). If alternative splicing was the mechanism by which multiple calpastatin mRNAs were produced, it is likely that there would be tissue specific expression of only one isoform of

mRNA and the several poly(A) addition sites would not be seen in the cDNAs isolated, as the alternative poly(A) site tends to be in the discarded exon (204,205). These observations tend to discount the possibility that the various calpastatin mRNAs are produced by alternative splicing. Isolation of the calpastatin gene will greatly aid the search for a possible multiple splicing pattern of exons, if they exist.

Several genes have been shown to produce multiple mRNAs with different lengths of 3' non-coding sequence (206-209). The mouse dihydrofolate reductase gene produces seven mRNAs in a range from 750 to 5600 nucleotides (206). Two mRNAs are produced from both the single β2-macroglobulin and chicken vimentin genes which both have mRNAs that differ in the length of their 3' noncoding sequence (208,209). It is likely that the different sizes of mRNA for calpastatin are the result of an analogous effect as that seen in the chicken vimentin mRNAs (208), in which there are no detectable exon junctions in the 3' non-coding region and in the longer isoform the shorter mRNA's AATAAA sequence is present. The expression of the two vimentin mRNAs is not equal in cells although they originate from the same gene (209). Unequal expression is also seen in the three isoforms of calpastatin mRNA in bovine skeletal muscle. The mechanism by which the poly(A) site is selected in these multiple mRNAs is not known but it is presumably the most efficient one which is chosen. Factors have been isolated which mediate the cleavage and poly(A) addition reactions (204). Recent work has suggested that the stability of interaction of these factors with the poly(A) site, particularly downstream sequence elements, play a role in determining the efficiency with which the poly(A) site is used (210).

5.3. The Bovine Trial.

5.3.1. The Effects of the β-Agonist Cimaterol on Calpain and Calpastatin Activities.

The growth data and the activities of the calpain system showed similar effects to those seen in previous studies in our laboratories (114 and unreported observations). There was an increase in L.dorsi muscle growth and protein deposition, measured as increased nitrogen (N) present, of the animals treated with the β-adrenergic agonist cimaterol (section 4.2.).

The effect on the activities of calpain system in the L.dorsi of the treated animals examined in this thesis was a significant increase in calpain II and calpastatin with a nonsignificant decrease in calpain I, Table 28 A. There were similar effects on the calpain II and calpastatin activities of L.dorsi in a β-agonist bovine growth trial carried out within our laboratories by Higgins et al (unpublished observations), Table 28 B. The β-agonist in this trial was also cimaterol which was administered via a subcutaneous osmotic minipump at a dose of 0.06mg/kg bodyweight/day whilst the animals were fed grass silage *ad libitum*.. Calpain I activity did not change significantly, but it increased, unlike the trial examined in this thesis. There were the usual changes in growth characteristics recorded, increased muscle weight and protein deposition in the L.dorsi. Comparisons with the studies in lambs show that β-agonists have analogous effects on their L.dorsi, Table 28 C and D (114,121). In the lamb trial carried out by Higgins et al (114) there was greater increase in calpain II activity than calpastatin. The variations in the activities observed in the four trials shown in Table 28 may have be due to the various β-agonists used having different potencies and the dose at which they were given.

Beta-agonist induced hypertrophy is believed to be mediated through the secondary messenger cAMP (115,120). The changes in activity of the calpains and calpastatin could be through direct phosphorylation. Murachi (130) has suggested calpastatin is a phosphorylatable substrate but the kinase involved was not positively

Table 28: Comparison of the effects of β-agonists on the activities of the calpain system components in the skeletal muscle(L.dorsi) of farm species.

	Agonist	Enzyme/Inhibitor	Activity Control	Activity Treated	% change	P
A.			(n=4)	(n=4)		
			(units/kg muscle)			
Bovine trial [1]	Cimaterol	Calpain I	1073	767	-29	NS
		Calpain II	813	1073	27	<0.05
		Calpastatin	2138	3759	76	<0.05
B.			(n=6)	(n=6)		
			(units/kg muscle)			
Bovine trial [2] (unpublished observations)	Cimaterol	Calpain I	213	243	14	NS
		Calpain II	827	1033	25	< 0.05
		Calpastatin	1910	3302	73	< 0.05
C.			(n=6)	(n=6)		
			(units/kg muscle)			
Lamb trial Higgins et al,(114)	Clenbuterol	Calpain I	570	490	-14	NS
		Calpain II	1250	2880	130	< 0.05
		Calpastatin	2740	5440	99	< 0.05
D.			(n=11)	(n=11)		
			(units/kg muscle)[a]			
Lamb trial Kretchmar et al,(121)	L-644,969	Calpain I	550	500	-10	< 0.05
		Calpain II	650	950	41	< 0.05
		Calpastatin	1250	2200	74	< 0.05

NS = non-significance p> 0.05

[a] These values were expressed graphically by Kretchmar et al using different definitions of units so the values shown are approximations

[1] Results of study described in this thesis.

[2] Higgins et al, unpublished observations from within our laboratories the details of which are outlined in the text (section 5.3.1.)

identified (section 2.5.1.). The effects of phosphorylation on activity are not known, but it appears to influence subcellular distribution which may result in altered recovery from cells and the activity measurements of the inhibitor. As described in section 2.5.1. calpain can also be phosphorylated in vitro (132). This was stimulated in the presence of cAMP and appeared to reduce the specific activity of the enzyme but had no effect on the calcium sensitivity. However Adachi et al (133) found that calpains would not undergo phosphorylation in vivo. The exact role of phosphorylation on the activity of the calpain system does require further investigation especially with respect to β-agonist mediated increases in cAMP and the possible subsequent possible events.

In farm species treated with β-agonists there is an increase in calpastatin activity (114,121), which in bovine L.dorsi is greater than combined activities of calpain I and II. Increasing the inhibitor's activity could be a means of decreasing the activity of the calpains thereby bringing about the β-agonist induced reduction in protein degradation. As cytosolic calpain II appears to be inactive at physiological calcium concentrations in vitro the effect of calpastatin would be to inhibit the physiologically active calpain I.

From recent observations on the activation characteristics of the calpains in their native and autolytically activated forms and the interaction with calpastatin, the calpain system appears to be more complex than the simplistic model outlined above, reviewed in sections 2.1.2. and 2.2.3..

In vitro calpastatin has different affinities for calpain according to the isoform's autolytic state. From the observations of various research groups, calpain II interaction with the membrane and the resulting autolysis is a possible means by which enzyme can increase its calcium sensitivity. The inhibitor also interacts with the membrane. So augmented calpain II activity at the calcium concentrations present in vivo may be possible, but increases in the inhibitor may have a buffering effect preventing large elevations in the calpain II activity at the membrane and also in the cytosolic activity of the membrane-dissociated autolytically activated form. The increased calpain II activity may be a result of the need for membrane reorganization in growing cells as many of the substrates for calpain are membrane-associated proteins, some of which are cytoskeletal.

The interaction of calpastatin with calpain I may be the direct inhibition of the enzyme in the cytosol where it could be active at the higher range of physiological calcium concentrations. As described in section 2.1.2. the calcium dependence for activation of native calpain I and that required for inhibition is similar. Augmented inhibitor activity could also prevent calpain I's association to the membrane as this appears to be through its binding to membrane associated substrate proteins with which the inhibitor could be competitive.

Although the above mechanisms are speculative, the overall action of β-agonists appears to be the reduction of protein degradation. The effects on the calpain system and the apparent reduction in their potential proteolytic action appear to fit this observation. There are other cytosolic proteinases which have been implicated in states of skeletal muscle atrophy as have the calpains (82). The cytosolic proteinases operate by various mechanisms such as the ubiquitin-conjugated ATP-dependent/independent system like the multicatalytic enzyme megapain. Others are ATP-dependent or ATP-stimulated proteolytic enzymes like the multicatalytic proteinase (1,2,211). What role the other cytosolic proteinases play in β-agonist induced protein deposition is not known.

The effects of the β-agonist induced skeletal muscle hypertrophy on the activities of the lysosomal cathepsins have also not been studied in any great depth (section 2.4.0.). These enzymes have a low pH optimum for activity and are unstable at neutral and alkaline pH so have to be compartmentalized in the acidic environment of the lysosomes (1,203). Although they have a definite role in proteolysis they probably require the 'loosening' of muscle myofibril structure by cytosolic enzymes, such as calpain, to become involved.

5.3.2. The Effects of the β-Agonist Cimaterol on Gene Expression. Extracted total RNA and its <u>in vitro</u> translation.

The quantity of intact total RNA extracted was not significantly different in the L.dorsi of treated and control animals expressed per gram of muscle (section 4.2.2.). Although the method of extraction was semi-quantitative, unlike the chemical digestion

analysis of total RNA by the modified procedures originally described by Munro and Fleck (212), the results obtained from extractions were reproducible so comparisons are valid. Smith et al (213) who extracted intact total RNA during their examination of the effects of β-agonists on specific L.dorsi mRNAs, also observed no significant effect of the agonist ractopamine on the quantity of RNA present in treated and control animals, which was in the range of 40-60μg/g muscle.

Wu et al (214) had observed that the quantity of intact total RNA extracted decreased in the heifers given β-agonists, whilst there was no difference in the amount of 'translatable' mRNA. This suggested that the poly(A)+ RNA made up a greater proportion of the total RNA in the treated animals. However, they did not quantify the poly(A)+ RNA directly.

The observations in this thesis indicated there was no difference in the quantities of the total RNA extracted. There was also no significant change in the in vitro translation capability of the isolated L.dorsi total RNA (section 4.2.3.). Therefore, according to the rationale of Wu et al (214), there was probably no change in the proportion of poly(A)+ RNA in the L.dorsi total RNA. This observation was confirmed by Smith et al (213), who isolated the poly(A)+ RNA from L.dorsi total RNA of β-agonist treated steers.

Analysis of in vitro translation products on SDS-PAGE from the total RNA of the bovine trial did not show any major shifts in level of mRNA expression indicated by increased protein products, nor in the types of proteins expressed (Figure 42).

Examination of the total RNA on denaturing agarose gel electrophoresis failed to show any qualitative changes in the total RNA isolated which may have suggested an increase in non-mRNA species such as rRNA. Comparison of the 28S and 18S rRNA intensities on ethidium bromide stained gel by scanning densitometry failed to show any difference in their relative expression (section 4.2.2.). This estimate was a semi-quantitative indication of whether the proportion of rRNA in the total RNA had increased as the concentration of extractable total RNA had remained the same in the control and

treated animals. There appeared to be no difference between the samples suggesting no major shifts in the types of RNA expressed in the isolated total RNA.

Several groups have reported β-agonist induced increases in skeletal muscle total RNA expressed as per gram protein or gram muscle (120,116,216,217). These observations were based on the analysis of total RNA by chemical digestion (212). They gave no indication whether any specific mRNAs increased and whether there were changes in the RNA making up the translation machinery i.e. the quantity of rRNA and tRNA. Analysis of the DNA content has not shown any increase in the β-agonist treated animals (216-218). Beerman et al (216) and Kim et al (218) suggested that satellite cells were not required to proliferate and be incorporated into skeletal muscle myotubes for there to be increased growth in the treated animals, although this is seen in certain types of muscle hypertrophy (219,220). The increase in skeletal muscle RNA suggests an increase in the capacity for protein synthesis. Various research groups, from their experimental evidence, have suggested that protein synthesis is also increased in β-agonist induced hypertrophy (115,118,217).

To produce extracted total RNA results in the loss of some RNA species. The quantification of changes in total RNA by chemical means does give different results to those observed for extracted total RNA, but these are not directly contradictory.

α-actin and myosin light chain 2 mRNA expression.

Using the two myofibrillar protein cDNAs, α-actin and myosin light chain 2 (MLC2), the total RNA was examined for changes in their mRNA expression to give an indication whether there was an increase in the translational capacity for these proteins in the bovine L.dorsi (section 4.2.4.). Northern blot analysis showed significant increases in the expression of both the mRNAs between control and cimaterol treated animals. When the analysis was repeated using slot blotting there was no apparent difference between controls and cimaterol treated animals for both mRNA species over the linear part of the hybridization intensity curve. However, the samples from the treated animals always had a greater hybridization signal than the controls at higher loadings of total RNA.

The discrepancy between the two methods of analysis is probably due to uneven Northern blotting as a large gel was used to accommodate all the samples. The quantity of total RNA used (25μg) probably meant that hybridization was nonlinear, as was the case in the larger quantities loaded on the slot blot. Although the two probes were not specific to bovine skeletal muscle they did cross hybridize, Figure 22 and 23. The mouse α-actin cDNA was the better of the two hybridizing at a high stringency than the chicken MLC2 cDNA. This was expected as actin is a conserved protein across species as well as there being less evolutionary divergence between mice and the steer samples compared with chickens and steers.

The slot blots indicated that there was no apparent large increase in skeletal muscle α-actin and MLC2 mRNA in the cimaterol treated L.dorsi samples. However at the higher loadings of total RNA there was a greater hybridization signal in the cimaterol samples. This may have been caused by nonspecific hybridization as it was observed in the nonlinear region of the relationship between total RNA loaded and hybridization signal intensity (see Figures 47, 49 and 50) . The observations from the slot blots and the lack of any notable increase in the muscle ^{35}S-labelled protein products from total RNA in vitro translation suggests that here is no major augmented expression of the muscle protein mRNA transcripts due to cimaterol treatment in the bovine L.dorsi.

The increases observed in total RNA when it is analysed by chemical digestion in other laboratories may be in non-mRNA species. By augmenting translational efficiency (increasing the quantity of protein synthesised per unit mRNA) translational capacity could remain unaltered (units mRNA per unit protein). The small increases in the level of mRNA for MLC2 and α-actin, seen in the slot blots (section 4.2.4.), although apparently insignificant could be sufficient to increase the synthesis of protein enough to be significant at a post-translational level.

Smith et al (213) have also examined the effect of β-agonists on specific mRNAs in bovine skeletal mRNA. They used the β-agonist ractopamine which they fed to steers at various doses. After extracting total RNA they probed it with a bovine myosin light chain 1/3 (MLC 1/3) cDNA. A significant increase in the intensity of the hybridization

signal for probed slots in the ractopamine treated animals was observed. However single slots of 5µg total RNA were used for their analysis which may have encountered the problem of nonlinear hybridization. Also the cDNA probe used had not been fully characterised and did not appear to hybridize specifically in Northern blots (213).

Babij and Booth (221) examined the effect of clenbuterol administration to rats on the expression of α-actin mRNA in atrophying skeletal muscle caused by denervation and limb suspension. They found that the 60% decrease in α-actin mRNA seen in denervation was prevented by the administration of the β-agonist. However, the complexity of the relationship between muscle atrophy and the effects of clenbuterol compared to β-agonist induced muscle hypertrophy makes it difficult to draw related conclusions. In the recovery of the mRNA expression in atrophy the mRNA is induced back to the 'normal', whilst in hypertrophy it would have to be induced above this normal level which may be effected by innervation.

Examination of the effects of β-agonists on the expression of myofibrillar proteins' mRNA requires careful analysis of cDNA probe hybridization data determined from the linear part of the relationship between hybridization intensity and quantity of RNA blotted. To achieve this the concentration of the probe used should be in excess of membrane bound target sequences and the cDNA should ideally be specific to the sequence being probed. Homology between the chicken and bovine MLC2 sequence is sufficient for hybridization between the cDNA and the target bovine mRNA. The MLC2 cDNA probe did hybridize to a specific RNA species on Northern blots, although high stringency conditions were not used. The lack of complete homology between species does not effect the use for comparison between treatments although it does appear to produce more erratic values compared with the apparently higher homology probe α-actin (section 4.2.4.).

Calpain mRNA.

The human calpain II large subunit cDNA probe was effective against bovine skeletal muscle total RNA whilst the human calpain I large subunit cDNA was not, for the reasons outlined in section 4.1.4..

The level of Calpain II large subunit mRNA did increase (34%) in the L.dorsi of the cimaterol treated animals (section 4.2.5.). Although this was not significant it is comparable with the elevated proteolytic activity (27%) which was significant (section 4.2.1.). In order to confirm whether calpain II large subunit gene expression is induced by β-agonists a system where a greater response in enzyme activity is observed to the agonist needs to be examined, for example in lambs where the increase in calpain II activity exceeded that of calpastatin (Table 28 C), using species and isoform specific probes.

The nonsignificant increase in cimaterol induced expression of calpain II large subunit mRNA would be expected from the data available on the sequence of the human calpain II large subunit gene (159). The promoter region of this gene appears not to contain a cAMP responsive element (CRE) or a AP2 binding site (section 2.5.3.). As β-agonists are believed to mediate their growth effects on skeletal muscle through cAMP the calpain II gene would not be expected to show increased expression.

Although the promoter of the calpain II large subunit gene does not appear to contain any elements that are known to respond to cAMP the small increase in its mRNA could be through 'secondary' mechanisms. The mRNA expression from the c-jun and c-fos genes have been shown to be induced by β-agonists in rat neonatal myocardial cells (223) and c-fos mRNA expression was increased in various cell types including the rat submandibular gland (162) and BC3H1 nonfusing muscle cells (224). The products of c-fos and c-jun genes form heterodimers that bind to AP1 sites which are known to regulate gene transcription (222). The promoter region of the calpain II large subunit gene does contain a putative AP1 binding site. Several recent observations have shown that a cAMP responsive element binding protein (CREB) will form a dimer with a gene product of the jun family (152,153). The Fos-Jun protein heterodimers can bind to CREs suggesting the capacity for transcriptional 'cross talk' in these systems (225,226). Therefore it can be speculated that a low level of increased calpain II large subunit gene expression could take place by this β-agonist induced expression of c-fos and c-jun mRNA, which in turn activates the AP1 site in the gene's promoter. However, as with

most promoter sequence motifs, the AP1 site reported is a putative one and there is no direct evidence for the stimulation of gene transcription through AP1 binding sites via an indirect mechanism stimulated by cAMP, so this mechanism is speculative. AP1 binding sites, alternatively called a phorbol ester (TPA)-responsive element (TRE), have been identified in other genes by their ability to induce gene transcription via phorbol ester stimulation of cells (222). The calpain large subunit gene requires careful examination in order to determine whether the gene can be induced by phorbol esters through this AP1 site.

Although other promoter elements have been identified in the calpain II large subunit gene, such as an Sp1 recognition site and one characteristic of the β-globulin gene, the presence of a putative AP1 is of interest because of calpain's identified role in the down-regulation of protein kinase C in vivo (227), which is also activated by phorbol esters. A putative AP1 binding site has also been identified in the calpain small subunit (160) suggesting some degree of co-ordinate regulation of the two subunits which may be linked to protein kinase C regulation.

Hata et al (159) suggested that the calpain II large subunit gene promoter region was characteristic of a 'housekeeping' protein. The gene appeared to be under the influence of negative regulatory elements 5' to the immediate promoter region to the gene, the implication being that there is tight control over the expression of calpain II large subunit. They suggested that because of the ubiquity of calpain distribution expression is possibly controlled by the negative regulatory promoter elements, which are regulated by a negative trans-acting factor. The expression of this factor would be expected to be cell specific thereby accounting for varying degrees of expression of calpain activity in different tissues, the housekeeping promoter being responsible for the ubiquitous tissue expression (9). Although this hypothesis suggests that the induction of calpain II large subunit gene transcription by secondary messengers, such as cAMP or through agents like phorbol esters is unlikely, Hata et al (159) did not examine the effects of agonists on cells transfected with the calpain II large subunit gene. Calpain I and II mRNA has been shown to be induced 4-6 fold in the biceps femoris of fasting rabbits so there must be

some mechanism by which the genes can be significantly induced (228). However the role of the proteinase would presumably be different in a state of muscle atrophy than that seen in β–agonist induced hypertrophy where there is a selective increase in only the calpain II isoform activity and the possible increase in the mRNA expression observed in this thesis has not been fully elucidated.

The calpain I large subunit gene has not been isolated, therefore the possible relationship of increases in intracellular cAMP to its expression is not known. Further work is required to see if its mRNA expression is affected by β-agonists. The type of promoter/enhancer sequences in the 5' flanking region of the gene would be expected to be different to the calpain II large subunit gene because of its own distinct level of protein expression compared to calpain II; for example in erythrocytes calpain I activity is expressed whilst calpain II activity is absent (9). This is an indication that there may be a different response of the calpain I large subunit gene to the β–agonists.

As small subunits are required for the activity of both calpain isoforms then some common promoter sequences would be expected in both calpain I and II large subunits and small subunit to allow coordinate regulation. Similarities have been shown between the promoter regions of the calpain II large subunit and the small subunit genes (159). The effects of β–agonists on the gene expression of the small subunit also requires examination.

The work carried out in this thesis failed to show a significant increase in calpain II large subunit mRNA in L.dorsi in response to β–agonist treatment. Although the augmented calpain II activity may be due to increased gene expression the effects could be caused by cAMP-induced phosphorylation of the enzyme altering its activity. Adachi et al (133) observed no phosphorylation of the enzyme <u>in vivo</u> but they did not examine the effects of stimulating cells with various agonist. The involvement of phosphorylation as a means of altered calpain activity requires further investigation.

The means by which calpain II activity is significantly increased in the skeletal muscles of β-agonist treated animals, as observed in other experiments carried out in our laboratories (114), has yet to be explained.

Calpastatin mRNA.

From Northern blot analysis of L.dorsi cimaterol and control total RNA samples there was a significant increase in the intensity of the 3.7-3.8kb hybridization signal (161%) (section 4.2.6). The increase was greater than the change in the inhibitor activity (76%) (section 4.2.1.). Similar effects were seen in streptozotocin induced diabetes where the expression of the mRNA to G_i-1 in adipocytes was induced four fold whilst the protein increased by a factor of two (196).

The increase in the calpastatin 3.8kb mRNA could be due to augmented gene(s) transcription or effects on the stability of the mRNA induced by cimaterol, or both. As the three mRNAs produced from the calpastatin gene are likely to be the result of different poly(A) addition site selection (section 5.2.), if increased gene expression causes the significant increase in only the 3.8kb isoform this has to be achieved by differential selection of one poly(A) addition site above the others, assuming that the mRNA is not being selectively stabilized over the other calpastatin mRNAs. Augmented transcription of a single calpastatin gene without a selection mechanism for a particular poly(A) site would be expected to result in the three mRNAs increasing but remaining in the same expression ratio seen in the unstimulated state.

The alternative explanation is that cimaterol treatment induces increased stability of the 3.8kb mRNA thereby elevating its expression. This may be acting in concert with the changes in the gene expression. The hybridization signal for all three calpastatin mRNAs did increase, although not significantly for all of them, but by preferentially stabilizing the 3.8kb species its expression would be further enhanced above the others making its increase significant.

Similar effects on the level of transcription and the stability of mRNA have been shown to be induced by cAMP (section 2.5.2). Increased expression of calpastatin mRNA could be similarly mediated as its gene expression is clearly affected in cimaterol stimulated bovine skeletal muscle hypertrophy. In order to directly respond to cAMP the gene(s) would be expected to contain a cAMP responsive element, either a CRE or an AP2 binding site. It is not known whether the 5' flanking region of the gene(s) contains

such elements. If there is increased expression of the gene by cAMP-mediated events then the second messenger probably also has an effect on either the factors involved in the selection of poly(A) sites or those determining the stability of the particular mRNA species as one is preferentially selecting over the others. This presumably is achieved by interaction with a sequence located at the particular poly(A) addition site or in the 3' noncoding region of the mature mRNA in question.

Most of the genes that have identifiable CREs are rapidly induced by cAMP within minutes of stimulation and are classified as group 1 responsive genes (134). The rat cystatin S gene in the submandibular gland only responds after a relatively longer period of four hours after the administration of a β-agonist (229) and is categorised in group 2, the slower responding genes (134). This suggests that the group 2 genes require the synthesis of factors for the effects of cAMP to be mediated on transcription. The group 1 genes probably need only the modification of already existing factors by cAMP mediated events.

The gene expression of calpain activity appears to be under tight regulation. Its activity could be considered as constitutively expressed, a 'housekeeping protein'. Calpastatin gene expression appears to be under the regulation of secondary messenger cAMP, which is known to induce rapid gene response as well as long term effects as seen in the hypertrophy of the rat submandibular gland (162). Alterations in calpastatin activity could be increased or decreased depending on the requirement of calpain mediated proteolysis by the induction of its gene expression through different secondary pathways to those of calpain. The time course for the response to β-agonist and its relationship to the expression of calpastatin and calpain activity is not known. This may be the key to the understanding of the role of cimaterol induced increased expression of calpastatin mRNA.

The biological significance of the various sizes of mRNA for calpastatin is not known. The single chicken vimentin gene has been shown to express two mRNA species which differ in the length of their 3' untranslated regions (208,209). Tissue specific differences in the relative expression of the two mRNAs suggests that the

selection of the poly(A) site has biological significance. Capetanaki et al (209) proposed that the different 3' untranslated region may affect the subcellular compartmentalisation of the mRNA and thereby the location of protein expression, or alternatively the stability of the mRNA could be affected, or both.

The directed compartment expression of calpastatin activity would affect its ability to inhibit calpain mediated proteolysis. By selecting a mRNA species calpastatin synthesis may be shifted to a particular region of the cell. Calpain activity, especially calpain II, could be considered to be functionally compartmentalised in an membrane environment as it is required for autolysis inorder to achieve activation at physiological calcium concentrations (section 2.1.2.). The effects of β-agonists may be to induce a change in the calpain to calpastatin ratio within a specific compartment thereby decreasing proteolytic potential.

5.4. Summary.

The exact roles of calpain I and II in skeletal muscle and their scope for activity in vivo as well as their interactions with calpastatin are not known. From in vitro studies it appears that the activation mechanism for the two isoforms is different (section 2.1.2.). Calpastatin has been shown to have distinct affinities for calpain I and II according to their autolytic state and location in the cell (section 2.2.2.), which may be influenced by post-translational modification.

One of the effects of β-agonists on skeletal muscle is believed to be a decrease in protein degradation (section 2.4.0.). This would be expected to be as a result of inhibiting the proteolytic activity within the muscle myofibrils. Calpain and calpastatin activities have clearly been shown to be altered in β-agonist treatment of farm species (section 2.4.0.). There is an increase in the activity of the inhibitor calpastatin which is specific to the calpains, which have been implicated in muscle myofibrillar protein turnover. The changes in the calpain system are toward increased inhibitory capacity and a change in the status of the calpain isoforms' activity. Although calpain II can increase its calcium sensitivity in vitro, studies suggest that it is not active at physiological calcium

concentrations. Autolysis at the membrane decreases the calcium requirement for activation (section 2.1.2.), therefore changes in the activity may be localised and required for proteolytic mediated rearrangement of cytoskeletal proteins in order for growth to occur or down regulation of membrane associated enzymes and receptors, such as protein kinase C (227).

The changes in calpain II activity may be induced by increased gene expression. There was an increase in the mRNA for calpain II large subunit (section 4.2.5.), but this was nonsignificant although it matched the increase in the enzyme activity (section 4.2.1.). This augmented calpain activity may be caused by a cAMP stimulated phosphorylation of the protein, a post-translational event. However, calpains have not been conclusively shown to be phosphorylated in vivo (133).

The elevation in calpastatin activity does appear to be due to increases in the expression of its mRNA, one species (3.8kb) being increased over the others (section 4.2.6.). Whether this is caused by increased gene transcription or by modifications of post-transcriptional events mediated by β-agonists through cAMP is not known.

There appears to be no significant change in the mRNAs capable of producing contractile proteins or increases in the level of specific mRNAs from the examination of in vitro translation products and specific myofibrillar protein mRNA (section 4.2.2., 4.2.3. and 4.2.4.). Although the quantity of extractable total RNA did not change on treatment with cimaterol the observed increases in total RNA by other groups is not incompatible with the present results for reasons outlined in section 5.3.2.. Elevated total RNA observed could be due to an increase in the RNA required for the translational machinery, thereby giving β-agonist treated animals a higher translational capability.

5.5. Further Work

Information on the number and the sequence of the 5' flanking region of the calpastatin gene(s) is required in order to interpret the observations in this thesis. Identification of the promoter elements is necessary in order to understand what trans-acting factors could be affecting gene transcription. The entire cDNA to bovine skeletal muscle calpastatin is also required to confirm whether there are multiple poly(A) addition site sequences in the 3' untranslated region as in the rabbit sequence (52).

The effects of β-agonists on the mRNA expression of the calpain isoforms needs to be repeated with specific probes for the species analysed using a more sensitive assay technique, such as S1 nuclease hybridization. In order to interpret completely information from such studies a detailed understanding of gene structure for both isoforms of calpain large subunits as well as the small subunit will have to be gained, as is required for calpastatin, so that the possible trans-acting factors can be identified.

Cyclic AMP inducible genes respond relatively rapidly to the secondary messenger (134). As outlined above calpastatin may be the component of the calpain system which is inducible so that it can regulate the activity of calpain. The increase in expression of calpastatin mRNA that is mirrored by an increase in activity in response to β-agonist (presumably acting through cAMP which is known to rapidly induce gene transcription by phosphorylation of trans-acting CREBs) further suggests that calpastatin is the controlling factor over the reported 'housekeeping' calpains (159). The time course of the calpain system's response to β-agonists needs to be carried out so that rapidity of the induction of calpain II and calpastatin activity and mRNA expression can examined and this hypothesis tested.

The role of activators on the interaction of the calpains to calpastatin and their effects in altering the calcium sensitivity of calpain II may be another means of changing calpain activity without altering calpain or calpastatin gene expression. The activators are reported to increase calpain II sensitivity to calcium and also decrease the inhibitory potential of calpastatin. Alternatively changes in activity may be mediated through direct phosphorylation of the enzymes and inhibitor via cAMP-dependent protein kinase. What

role this plays in inhibitor activity is not known, although some preliminary investigations have been carried out by Murachi et al (130) which suggest it effects the subcellular distribution of the inhibitor (section 2.5.1.). Further investigations are required on the post-translational mechanisms by which calpain and calpastatin activity could possibly be regulated

The β-agonist induced hypertrophy has been described as being specific to skeletal muscle (115), although hypertrophy effects have been seen in the rat submandibular gland and cultured neonatal myocardial cells. An increase in mRNA for the calpain I and II large subunits was observed to be specific to the skeletal muscle of fasting rabbits (228). Whether the β-agonist induced changes in the calpain systems activity associated with skeletal muscle hypertrophy is also muscle specific requires investigation. If this is the case then there must be some tissue specific factor that regulates its expression in response to β-agonists, as both calpain and calpastatin are ubiquitously expressed and the β-agonist induced cAMP is a common secondary messenger in most cells. The understanding of the regulation of the calpain system at the level of gene transcription will be as at least as difficult as the that of its protein activity.

Although the information available on the calpain system has expanded in the last five years, particularly with the isolation of the cDNA sequences, there is still little known about the physiological role of the enzymes and inhibitor or the activation mechanisms which take place in vivo. The use of specific calpain inhibitors, i.e. peptide fragments of calpastatin, has recently been employed in vivo (227) and will be a means by which calpain's physiological role can be partially determined. The understanding of the implications of altered calpain and calpastatin activity in β-agonist induced skeletal muscle hypertrophy may allow the more efficient manipulation of animal growth by the exploration of such promoters.

Appendices.

Appendix A.

cDNAs Used for Probe Hybridization.

i) Human Calpain I Large Subunit cDNA.

The plasmid p42, containing part of the human calpain I large subunit cDNA, was a gift from Dr.P.C.Emson, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge.

The calpain I large subunit cDNA was a Pst I fragment of the complete cDNA of the clone p31 in vector pUC8 (16). The calpain I insert was subcloned into pBR322 to give p42 which contains part of the coding nucleotide sequence and some of the 3' noncoding region extending from 1252 to 2517bp of the original cDNA in p31, Figure 60.

Calpain I cDNA insert of p42 was removed by Pst I restriction endonuclease digest to give the 1265bp fragment. The insert can be identified between the 1375 and 947bp fragments of the Eco RI/Hind III lambda DNA markers and was characterised by restriction endonuclease digest with Sma I and Pvu II, Figure 61 and 62. The predicted size of the Sma I digest fragments were 221, 961 and 83bp. It can be seen in Figure 61 that the Sma I digest gave a visible fragment slightly greater than that of 947bp of the Eco RI/Hind III markers. Pvu II digest of the p42 insert gave fragments less than 564bp (Figure 62), the predicted sizes were 406, 468, 107 and 284bp.

The Sma I and Pvu II sites are unique to the human calpain I large subunit cDNA insert and are not found in the similar sized human calpain II large subunit cDNA insert described below.

Figure 60: Plasmid map of p42. The map shows the position of the human calpain I large subunit cDNA insert relative to the complete sequence and the location of the relevant restriction endonuclease sites.

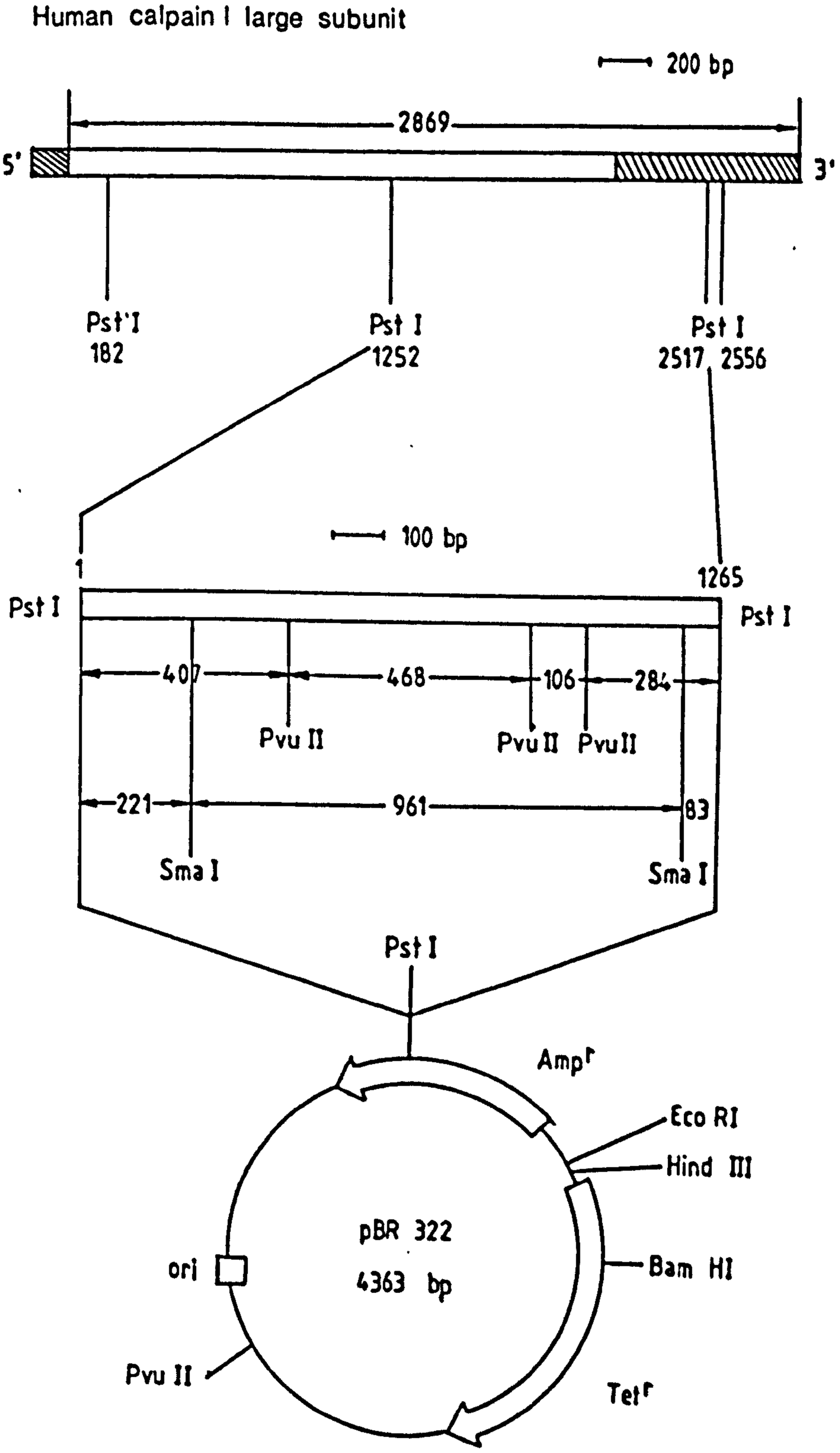


Figure 61: Nondenaturing 1% agarose electrophoresis of the human calpain I large subunit cDNA insert from p42 subjected to restriction endonuclease analysis. Lane 1 and 2 Eco RI/Hind III markers, lane 3 p42 insert digested with SmaI, lane 4 p42 insert, lane 5 and 6 Hind III markers.

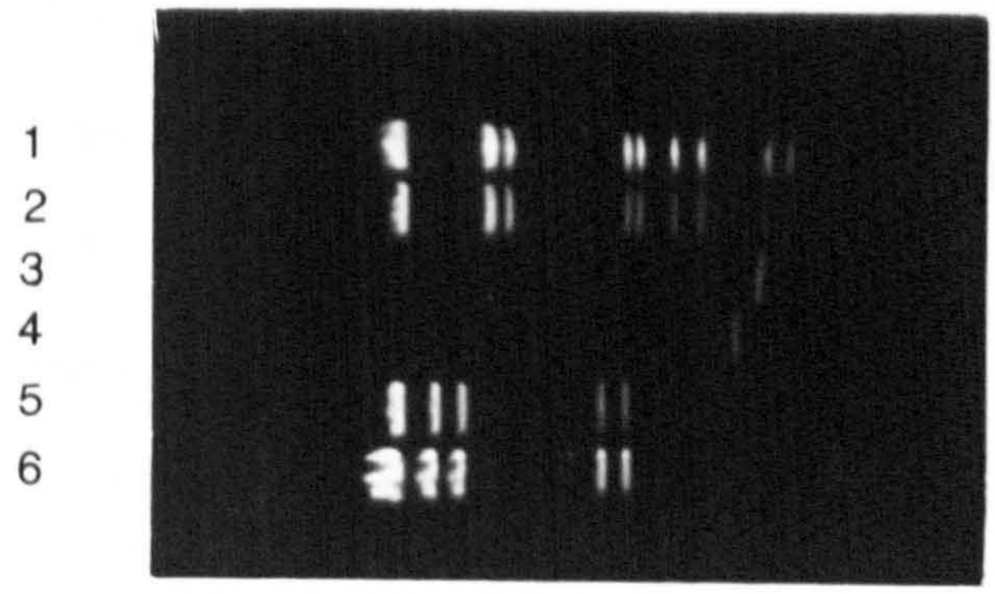
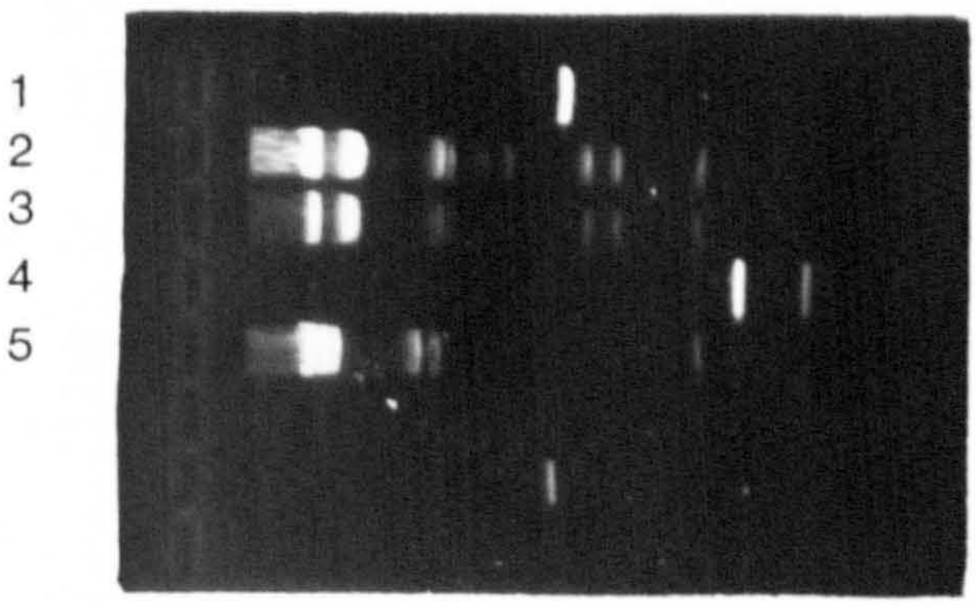


Figure 62: Nondenaturing 1% agarose electrophoresis of the human calpain I large subunit cDNA insert from p42 subjected to restriction endonuclease analysis. Lane 1 p42 insert, lane 2 and 3 EcoRI/Hind III markers, lane 4 p42 insert digested with Pvu II, lane 5 Hind III markers.



ii) Human Calpain II Large Subunit cDNA.

The plasmid p21-16, containing the calpain II large subunit cDNA, was a gift from Dr.P.C.Emson (see above).

Restriction endonuclease digest with EcoRI of the clone λHS1, containing the complete cDNA for human calpain II, gave a fragment with the sequence for a small part of the 3'end of the primary coding sequence and all of the 3'non-coding region, 1866-3083 of the complete cDNA (17). This was subcloned into the Eco RI site of plasmid pUC8, to give clone p21-16, Figure 63. The human calpain II cDNA insert of p21-16 is 1217 bp long and was excised by Eco RI, Figure 64.

Restriction enzyme digest of the p21-16 calpain II large subunit cDNA insert with Hind III was predicted to give fragments of 716, 292 and 192 bp. Likewise digest with Bam HI would give 748, 298 and 171 bp fragments. Analysis of the fragments from the two digests gave the large fragments predicted, the Bam HI fragment is slightly larger than the Hind III, both fragments are between the 831 and 564bp products of the Eco RI/Hind III lambda markers, Figure 64.

From the data produced on the restriction endonuclease digests of the cDNAs the similarity of the resulting fragments with those predicted suggested that the clones contained the cDNAs for human calpain I and II large subunits.

Figure 63: Plasmid map of p21-16. The map shows the position of the human calpain II large subunit cDNA insert relative to the complete cDNA sequence and the location of the relevant restriction endonuclease sites.

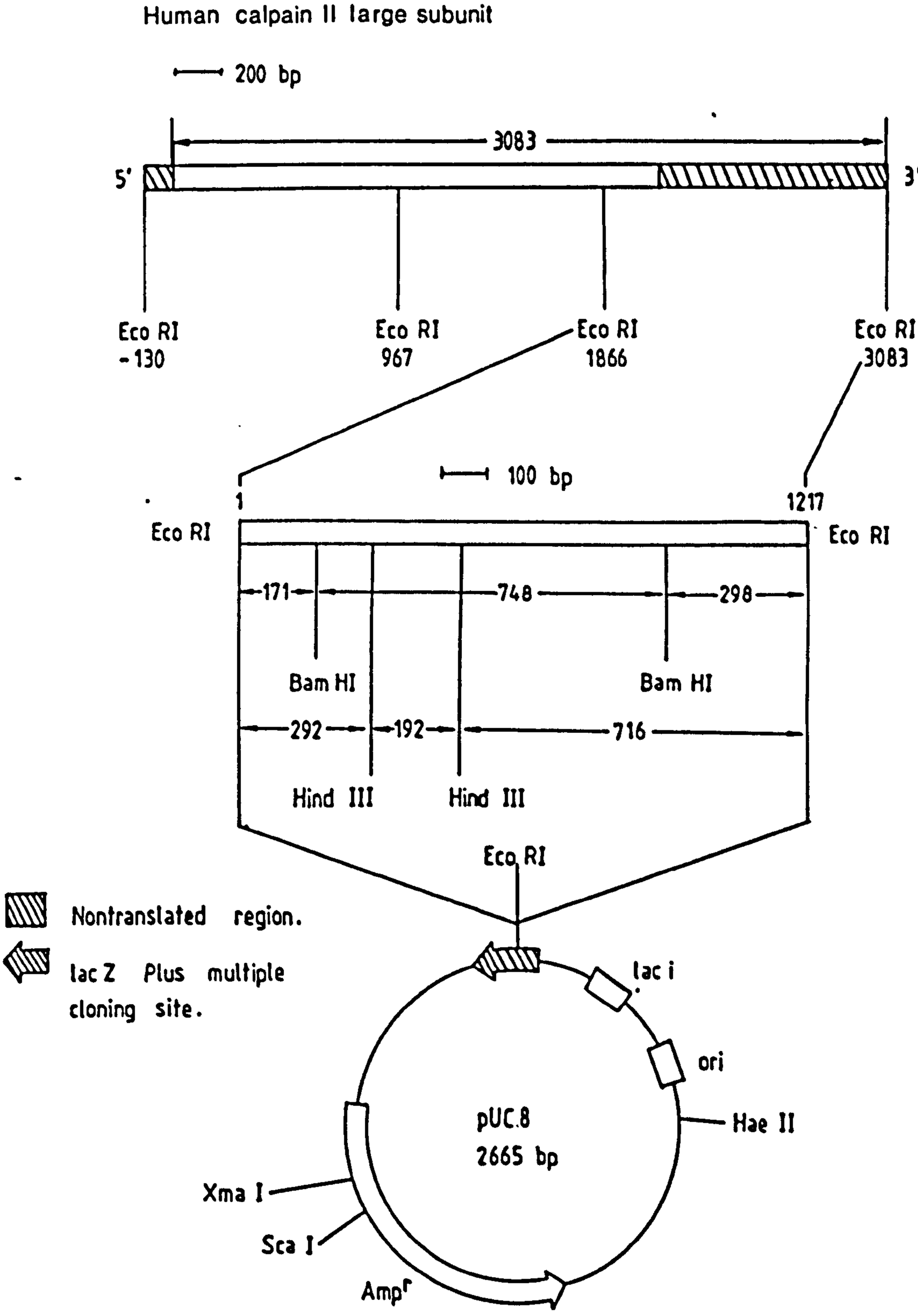
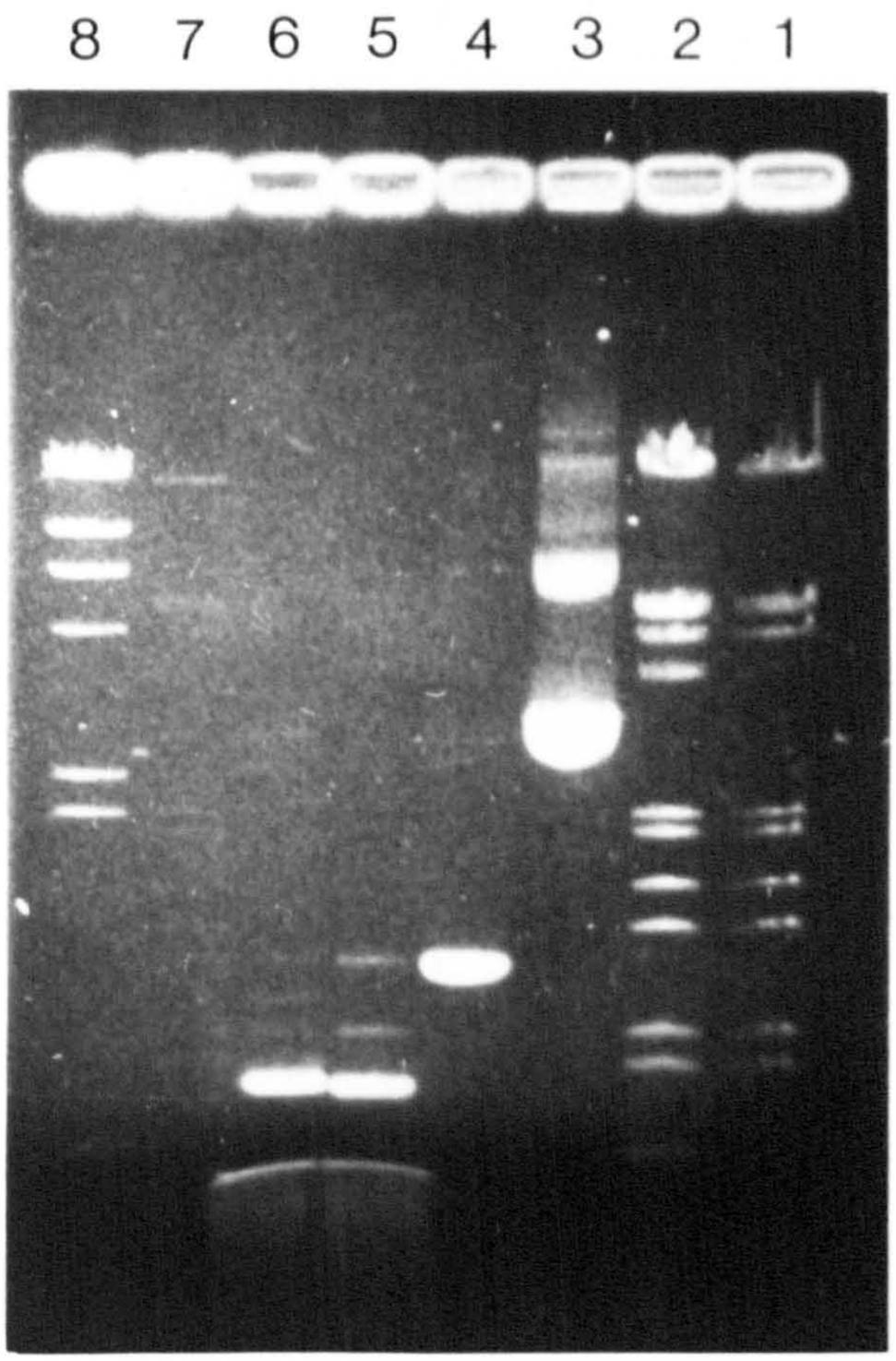


Figure 64: Nondenaturing 1% agarose electrophoresis of the human calpain II large subunit cDNA insert from p21-16 subjected to restriction endonuclease digest. Lane 1 and 2 Eco RI/Hind III markers, lane 3 plasmid p21-16, lane 4 p21-16 insert, lane 5 p21-16 insert digested with Hind III, lane 6 p21-16 insert digested with Bam HI, lane 8 Hind III markers.



ii) Mouse α-Actin cDNA.

The plasmid containing the α-actin cDNA, pAM91, was obtained through Dr. R.S.Gilmour, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge from Dr.A.Balmain, Wolfson Laboratory for Molecular Pathology, Beatson Institute for Cancer Research, Glasgow.

The plasmid was selected from a mouse skeletal muscle cDNA library cloned into vector pBR322 and contains a cDNA insert to α-actin, which is 1358 bp long (230). This was not the full length sequence for mRNA to α-actin, which is approximately 1650 bp. The clone pAM91 contains approximately 90% of the amino acid coding nucleotide sequence (88-1125 bp) of the complete sequence and part of the 3' non-coding sequence (1125-1358 bp) of the complete sequence. It is excised from pBR322 by Pst I restriction enzyme digest to give a cDNA fragment 1173 and 185bp (231), of which the longer of the two was isolated and used. The cDNA was characterised by restriction endonuclease digest carried out by K.Fairbrother within our laboratories.

iv) Chicken Myosin Light Chain 2 cDNA.

The plasmid containing the myosin light chain 2 (MLC2) cDNA, M13mpFXLC2, was a gift from Dr.F.C.Reinach obtained through Dr.J.Kendrick-Jones, MRC Laboratory of Molecular Molecular Biology, MRC Centre, Cambridge.

The vector λgt11/L10 containing the complete primary coding, 3'non-coding and part of the 5'non-coding sequence of chicken MLC2 was isolated from a cDNA library cloned into λgt11 (232). The cDNA insert of λgt11/L10 was subcloned into M13mp11FX to give plasmid M13mp11FXMLC2 (233), which contains the amino acid coding sequence and the 3' non-coding region of the chicken MLC2 cDNA. The M13mp11FX plasmid is an adapted M13mp11 plasmid containing a sequence coding for a recognition site of the blood coagulation factor Xa, called the FX site (234), into which the MLC2 cDNA sequence was linked when cloned into the plasmid (233).

The cDNA of MLC2 cDNA was excised from M13mp11FXMLC2 by restriction endonuclease digest with Hind III and Eco RI to give a fragment of 700 bp, which

included the FX recognition site. The cDNA sequence was characterised by restriction endonuclease digest carried out Y.Lasslett within our laboratories.

Appendix B.

Lambda DNA Markers Produced by Restriction Endonuclease Digest.

The size of the DNA fragments produced by various restriction endonuclease digests of lambda DNA (NBL) are shown below.

	Size of fragments produced by digest (kb)		
	Hind III (7 fragments)	EcoRI/Hind III (12 fragments)	Pst I (29 fragments)
1	23.130	21.226	11.501
2	9.416	5.148	5.077
3	6.682	4.973	4.749
4	4.361	4.268	4.507
5	2.322	3.530	2.838
6	2.027	2.027	2.556
7	0.564	1.904	2.459
8		1.709	2.443
9		1.375	2.140
10		0.947	1.986
11		0.831	1.700
12		0.564	1.159
13			1.093
14			0.805
15			0.514
16			0.448
18			0.339
19			0.264
20			0.247
21			0.216
22			0.211
23			0.200
24			0.164
25			0.150
26			0.094
27			0.087
28			0.072
29			0.015

Appendix C.

Commonly Used Solutions and Buffers.

20xSSC

3.0M sodium chloride
0.3M sodium citrate pH7

20xSSPE

3.6M sodium chloride
0.2M sodium phosphate
20mM EDTA pH7.7

10xTBE

0.89M boric acid
0.89M Tris
25mM EDTA pH8.3

10xMOPS

0.2M 3-[N-morpholino]-propane-sulphonic acid (MOPS)
50mM sodium acetate
10mM EDTA pH8.3

TE

10mM Tris HCl
0.1mM EDTA pH8.0, pH7.6, pH7.4.

Appendix D.

Media for E.coli Cultures.

LB broth

1.0% (w/v) Bacto-Tryptone (Difco)

0.5% (w/v) yeast extract (Difco)

0.5% (w/v) sodium chloride pH7.2

Autoclave then add the appropriate antibiotic from a x1000 stock (12.5mg/ml Tetracycline in 50% ethanol or 50.0mg/ml Ampicillin in water) when cool.

LB agar

LB broth plus 1.0% (w/v) Bacto-Agar (Difco)

Autoclave, cool to 50°C before adding the appropriate antibiotic from stock then pour into culture plates to a depth of approximately 3-5mm.

Minimal agar

0.6% (w/v) disodium orthophosphate ($Na_2HPO_4.7H_2O$)

0.2% (w/v) potassium dihydrogen phosphate (KH_2PO_4)

0.05% (w/v) sodium chloride

0.1% (w/v) ammonium chloride pH7.4

1.5% (w/v) agar (Difco)

in 950 mls

Autoclave, to 60°C and add the following;

10ml 20% (w/v) glucose

1ml 0.1M calcium chloride

1ml 1.0M magnesium sulphate

50µl 100µg/ml thiamine

Adjust the volume to 1 litre and pour into the culture plates.

References.

References.

1. Dice J.F. (1987) FASEB J. 1,349-357.
2. Rivett A.J. (1989) Biochem. J. 263, 625-633.
3. Dayton W.R., Reville W.J., Goll D.E. and Stromer M.H. (1976) Biochem. 15, 2159-2167.
4. Toyohara H., Makinodan Y., Tanaka K. and Ikeda S. (1985) Comp.Biochem.Physiol 81B, 573-587.
5. Mykles D.L. and Skinner D.M. (1983) J.Biol.Chem. 258, 10474-10480.
6. Mellgren R.L. (1980) FEBS Lett 109, 129-133.
7. Kawashima S., Nomoto M., Hayashi M., Inomata M., Nakamura M. and Imahori K. (1984) J.Biochem. 95, 95-101.
8. Murachi T., Tanaka K., Hatanaka M. and Murakami T. (1981) Adv.Enzyme Reg. 19, 407-424.
9. Murachi T. (1983) TIBS 8, 167-169.
10. Takano E., Park Y-H., Kitahara A., Yamagata Y., Kannagi R. and Murachi T. (1988) Biochem.Int. 16, 391-395.
11. Pontremoli S. and Melloni E. (1986) Annu.Rev.Biochem. 55, 455-481.
12. Ishiura S., Murofushi H., Suzuki K. and Imahori K. (1978) J.Biochem. 84, 225-230.
13 Wolfe F.H., Sathe S.K., Goll D.E., Kleese W.C., Edmunds T. and Duperret S.M. (1989) Biochim.Biophys.Acta 98, 236-250.
14. Ohno S., Emori Y., Imajoh S., Kawasaki H., Kisaragi M.and Suzuki K. (1984) Nature (London) 312, 566-570.
15. Emori Y., Kawasaki H., Sugihara H., Imajoh S., Kawashima S. and Suzuki K. (1986) J.Biol.Chem. 261, 9465-9471.
16. Aoki K., Imajoh S., Ohno S., Emori Y., Koike M., Kosaki G. and Suzuki K. (1986) FEBS Lett 205, 313-317.
17. Imajoh S., Aoki K., Ohno S., Emori Y., Kawasaki H., Sugihara H. and Suzuki K. (1988) Biochem. 27, 8122-8128.
18. Sakihama T., Kakidani H., Zenita K., Yumoto N., Kikuch, T., Sasaki T., Kannagi R., Nakanishi S., Ohmori M., Takio K., Titani K. and Murachi T. (1985) Proc.Natl.Acad.Sci.USA 82, 6075-6079.
19. Emori Y., Kawasaki H., Imajoh S., Kawashima S. and Suzuki K. (1986) J.Biol.Chem. 261, 9472-9476.
20. Ohno S., Emori Y. and Suzuki K. (1986) Nuc. Acids Res. 14, 5559.
21. McClelland P., Lash J.A. and Hathaway D.R. (1989) J.Biol.Chem. 264, 17428-17431.

22. Kawasaki H., Imajoh S., Hayashi H. and Suzuki K. (1986) J.Biochem. 99, 1525-1532.
23. Suzuki K. (1987) TIBS 12, 103-105.
24. Minami Y., Emori Y., Kawasaki H. and Suzuki K. (1987) J.Biochem. 101, 889-895.
25. Imajoh S., Kawasaki H. and Suzuki K. (1986) J.Biochem. 99, 1281-1284.
26. Minami Y., Emori Y., Imajoh-Ohmi S., Kawasaki H. and Suzuki K. (1988) J.Biochem. 104, 927-933.
27. Suzuki K. and Ishiura S. (1983) J.Biochem. 93, 1463-1471.
28. Sorimachi H., Imajoh-Ohmi S., Emori Y., Kawasaki H., Ohno S, Minami Y. and Suzuki K. (1989) J.Biol.Chem. 264, 20106-20111.
29. Sorimachi H., Ohmi S., Emori Y., Kawasaki H., Saido T.C., Ohno S., Minami Y. and Suzuki K. (1990) Biol.Chem.Hoppe-Seyler 371, suppl. 171-176.
30. Suzuki K., Imajoh S., Emori Y., Kawasaki H., Minami Y. and Ohno S. (1988) Adv. Enzyme Regul. 27, 153-169.
31. Thompson V.F., Goll D.E. and Kleese W.C. (1990) Biol.Chem. Hoppe Seyler 371, suppl. 177-185.
32. Cong J., Goll D.E., Peterson A.M. and Kapprell H. (1989) J.Biol.Chem. 264, 10096-10103.
33. Inomata M., Hayashi M., Nakamura M., Saito Y. and Kawashima S. (1989) J.Biol.Chem. 264, 18838-18843.
34. Inomata M., Saito Y., Kon K.and Kawashima S. (1990) Biochem.Biophys.Res.Commun. 171, 625-632.
35. Suzuki K., Tsuji S., Kubota S., Kimura Y. and Imahori K. (1981) J.Biochem. 90, 275-278.
36. Suzuki K., Tsuji S., Ishiura S., Kimura Y., Kubota S. and Imahori K. (1981) J.Biochem. 90, 1787-1793.
37. Imajoh S., Kawasaki H. and Suzuki K. (1986) J.Biochem. 100, 633-642.
38. Crawford C., Willis A.C. and Gagnon J. (1987) Biochem.J. 248, 579-588.
39. Coolican S.A. and Hathaway D.R. (1984) J.Biol.Chem. 259, 11627-11630.
40. Imajoh S., Kawasaki H. and Suzuki K. (1986) J.Biochem. 99, 1281-1284.
41. Garret C., Cottin P., Dufourcq J. and Ducastaing A. (1988) FEBS Lett 227, 209-214.
42. Melloni E., Pontremoli S., Michetti M., Sparatore B., Salamino F. and Horecker B.L. (1985) Proc.Natl.Acad.Sci. USA 82, 6435-6439.
43. Sakai K., Hayashi H., Kawashima S. and Akanuma H. (1989) Biochim.Biophys.Acta 985, 51-54.

44. Kuboki M., Ishii H. and Kazama M. (1990) J.Biochem. **107**, 776-780.
45. Pontremoli S., Viotti P.L., Michetti M., Sparatore B., Salamino F. and Melloni E. (1990) Biochem.Biophys.Res.Commun. **171**, 569-574.
46. Pontremoli S., Melloni E., Michetti M., Salamino F., Sparatore B. and Horecker B.L. (1988) Proc.Natl.Acad.Sci.USA **85**, 1740-1743.
47. Johnson P. and Hammer J.L. (1989) Comp.Biochem.Physiol. **94B**, 45-48.
48. Pontremoli S., Melloni E., Viotti P.L., Michetti M., Di Lisa F. and Siliprandi N. (1990) Biochem.Biophys.Res.Commun. **167**, 373-380.
49. Mellgren R.L. (1987) FASEB J. **1**, 110-115.
50. Nishiura I., Tanaka K., Yamato S. and Murachi T (1978) J.Biochem. **84**, 1657-1659.
51. Mellgren R.L. and Carr T.C. (1983) Arch.Biochem.Biophys **225**, 779-786.
52. Emori Y., Kawasaki H., Imajoh S., Imahori K. and Suzuki K. (1987) Proc.Natl.Acad.Sci.USA **84**, 3590-3594.
53. Takano E., Maki M., Mori H., Hatanaka M., Marti T., Titani K., Kannagi R., Ooi T. and Murachi T. (1988) Biochem. **27**, 1964-1972.
54. Asada K., Ishino Y., Shimaa M., Shimojo T., Endo M., Kimizuka F., Kato I., Maki M., Hatanaka M. and Murachi T. (1989) J.Enzyme Inhibition **3**, 49-56.
55. Adachi Y., Takano E., Murachi T. and Hatanaka M. (1988) Biol.Chem.Hoppe-Seyler **369**, Suppl. 223-227.
56. Uemori T., Shimojo T., Asada K., Asano T., Kimizuka F.,Kato I., Maki M., Hatanaka M., Murachi T., Hanzawa H. and Arata Y. (1990) Biochem.Biophys.Res.Commun. **166**, 1485-1493.
57. Imajoh S., Kawasaki H., Emori Y., Ishiura S., Minami Y., Sugita H., Imahori K. and Suzuki K. (1987) FEBS Lett **215**, 274-278.
58. Maki M., Takano E., Mori H., Sato A., Murachi T. and Hatanaka M. (1987) FEBS Lett **223**, 174-180.
59. Maki M., Takano E., Osawa T., Ooi T., Murachi T. and Hatanaka M. (1988) J.Biol.Chem. **263**, 10254-10261.
60. Kawasaki H., Emori Y., Imajoh-Ohmi S., Minami Y. and Suzuki K. (1989) J.Biochem. **106**, 274-281.
61. Maki M., Bagci H., Hamaguchi K., Ueda M., Murachi T. and Hatanaka M. (1989) J.Biol.Chem. **264**, 18866-18869.
62. Kapprell H-P. and Goll D.E. (1989) J.Biol.Chem. **264**, 17888-17896.
63. Mellgren R.L. (1988) Biochem.Biophys.Res.Commun. **150**, 170-176.
64. Mellgren R.L., Lane R.D. and Mericle M.T. (1989) Biochim.Biophys.Acta **999**, 71-77.
65. Nakamura M., Inomata M., Imajoh S., Suzuki K. and Kawashima S. (1989)

Biochem. 28, 449-455.

66. Mellgren R., Mericle M.T. and Lane R.D. (1986) Arch.Biochem.Biophys. 246, 233-239.
67. Nakamura M., Inomata M., Hayashi M., Imahori K. and Kawashima S. (1985) J.Biochem. 98, 757-765.
68. Reddy M.K., Rabinowitz R. and Zak R. (1983) Biochem.J. 209, 635-641.
69. Bullard B., Sainsbury G. and Miller N. (1990) J.Musc.Res.Cell Motility 11, 271-279.
70. Dayton W.R., Schollmeyer J.V., Lepley R.A. and Cortes L.R. (1981) Biochim.Biophys.Acta 659, 48-61.
71. Dayton W.R., Goll D.E., Stromer M.H., Reville W.J., Zeece M.G. and Robson R.M. (1975) in Proteinases and Biological Control (Reich E., Rifkin D.B., Shaw E., Eds) Cold Spring Harbor Conferences on Cell Proliferation, Vol.2, 551-577 Cold Spring Harbor, New York.
72. Pemrick S.M. and Grebenau R.C. (1984) J.Cell Biol. 99, 2297-2308.
73. Hu D.H., Kimura S., Kanashima S. and Maruyama K. (1989) Zoological Sci. 6, 797-800.
74. Dayton W.R. and Schollmeyer J.V. (1981) Exp.Cell Res. 136, 423-443.
75. Yoshimura N., Murachi T., Heath R., Newman G.R., Janasi B. and Kay J. (1986) Cell Tissue Res. 244, 265-270.
76. Ishiura S., Sugita H., Nonaka I. and Imahori K. (1980) J.Biochem. 87, 343-346.
77. Barth R. and Elce J.S. (1981) Am.J.Physiol. 240, E493- E498.
78. Wang K.K.W. (1990) TIPS 11, 139-142.
79. Bandalamente M.A., Hurst L.C. and Stracher A. (1989) Proc.Natl.Acad.Sci.USA 86, 5983-5987.
80. Rodemann H.P., Waxman L. and Goldberg A.L. (1982) J.Biol.Chem. 257, 8716-8723.
81. Zeman R.L., Kameyama T., Matsumoto K., Bernstein P. and Etlinger J.D. (1985) J.Biol.Chem. 260, 13619-13624.
82. Furuno F., Goodman M.N. and Goldberg A.L. (1990) J.Biol.Chem. 265, 8550-8557.
83. Dayton W.R., Schollmeyer J.V., Chan A.C. and Allen C.E. (1979) Biochim.Biophys.Acta 584, 216-230.
84. Kar N.C. and Pearson C.M. (1976) Clin.Chim.Acta 73, 293-297.
85. Reddy P.A., Anandavalli T.E. and Anandaraj M.P.J.S. (1986) Clin.Chim.Acta 160, 281-288.
86. Kawashima S., Nakamura M. and Hayashi M. (1990) Biol.Chem.Hoppe-Seyler

371, 205-210.

87. Rabbani N., Moses L. and Anandaraj M.P.J.S. (1987) Biochem.Med.Metab.Biol. 37, 282-286.
88. Johnson P. and Hammer J.L. (1988) Int.J.Biochem. 20, 1227-1230.
89. Morgan J.E., Hoffman E.P. and Partridge T.A. (1990) J.Cell Biol. 111, 2437-2449.
90. Turner P.R., Westwood T., Regen C.M. and Steinhardt R.A. (1988) Nature (London) 335, 735-738.
91. Hussain H., Dudley G.A. and Johnson P. (1987) Expl.Neurol. 97, 635-643.
92. Croall D.E., Morrow J.S. and DeMartino G.N. (1986) Biochim.Biophys.Acta 882, 287-296.
93. Harris A.S., Croall D.E. and Morrow J.S. (1989) J.Biol.Chem. 264, 17401-17408.
94. Harris A.S. and Morrow J.S. (1990) Proc.Natl.Acad.Sci.USA 87, 3009-3013.
95. Schollmeyer J.E. (1986) Exp.Cell Res. 163, 413-422.
96. Kaur H. and Sanwal B.D. (1981) Can.J.Biochem. 59, 743-747
97. Nelson W.J. and Traub P. (1983) Molec.Cell Biol. 3, 1146- 1156.
98. Johnson G.V.W., Jope R.S. and Binder L.I. (1989) Biochem.Biophys.Res.Commun. 163, 1505-1511.
99. Malik M.N., Ramaswamy S., Tuzio H., Shiekh A.M., Fenko M.D., Wisniewski H.M. and Howard R.G. (1987) Life Sci. 40, 593-604.
100. Billger M., Wallin M. and Karlsson J.O. (1988) Cell Calcium 9, 33-44.
101. Beckerle M.C., Burridge K., DeMartino G.N. and Croall D.E. (1987) Cell 51, 569-577.
102. Schollmeyer J.E. (1988) Science 240, 911-913.
103. Gates R.E. and King L.E. (1985) Biochem. 24, 5209-5215.
104. Lynch C.J., Sobo G.E. and Exton J.H. (1986) Biochim.Biophys.Acta 885, *110-120.*
105. Vedeckis W.V., Freeman M.R., Schrader *W.T. and O'Malley B.W. (1980)* Biochem. 19, 335-343.
106. Puca G.A., Nolan E., Sica V. and Bresciam F. (1977) J.Biol.Chem. 252, 1358-1366.
107. Murray A.W., Fournier A. and Hardy S.J. (1987) TIBS 12, 53-54.
108. Melloni E. and Pontremoli S. (1989) TINS 12, 438-444.
109. Farago A. and Nishizuka Y. (1990) FEBS Lett 268, 350-354.
110. Kishimoto A., Mikawa K., Hashimoto K., Yasuda I., Tanaka S., Tominaga M., Kuroda T. and Nishizuka Y. (1989) J.Biol.Chem. 264, 4088-4092.
111. Wang K.K.W., Villalobo A. and Roufogalis B.D. (1989) Biochem.J. 262, 693-

706.

112. Rogers S., Wells R. and Rechsteiner M. (1986) Science 234, 364-368.

113. Ballard R., Bardsley R.G. and Buttery P.J. (1988) Brit.J.Nutr. 59, 141-147.

114. Higgins J.A., Lasslett Y.V., Bardsley R.G. and Buttery P.J. (1988) Brit.J.Nutr. 60, 645-652.

115. Yang Y.T. and McElligott M.A. (1989) Biochem.J. 261, 1- 10.

116. Reeds P.J, Hay S.M., Dorwood P.M. and Palmer R.M. (1986) Brit.J.Nutr. 56, 249-258.

117. Bohorov O., Buttery P.J., Correia J.H.R.D. and Soar J.B. (1987) Brit.J.Nutr. 57, 99-107.

118. Williams P.E.V., Pagliani L., Innes G.M., Pennie K., Harris C.I. and Carthwaite P. (1987) Brit.J.Nutr. 57, 417-428.

119. Emery P.W., Rothwell N.J., Stock M.J. and Winter P.D. (1984) Biosci.Rep. 4, 83-91.

120. MacLennan P.A. and Edwards R.H.T. (1989) Biochem.J. 264, 573-579.

121. Kretchmar D.H., Hathaway M.R., Epley R.J. and Dayton W.R. (1989) Arch.Biochem.Biophys. 275, 228-235.

122. Kretchmar D.H., Hathaway M.R., Epley R.J. and Dayton W.R. (1990) J.Anim.Sci. 68, 1760-1772.

123. Wang S-Y. and Beerman D.H. (1988) J.Anim.Sci. 66, 2545-2550.

124. Forsberg N.E., Ilian M.A., Ali-Bar A., Cheeke P.R. and Wehr N.B. (1989) J.Anim.Sci. 67, 3313-3321.

125. McElligot M.A., Mulder J.F., Chaung L.Y. and Barreto A. (1987) Am.J.Physiol. 253, E370-375.

126. McElligot M.A., Mulder J.E. and Chaung L-Y. (1989) Comp.Biochem.Physiol. 92C, 135-138.

127. Bond J.S. and Butler P.E. (1987) Ann.Rev.Biochem. 56, 333-364.

128. Zeman R.J., Ludeman R. and Etlinger J.D. (1987) Am.J.Physiol. 252, E152-E155.

129. Rothwell N.J. and Stock M.J. (1985) Biosci.Rep. 5, 755-760.

130. Murachi T. (1989) Biochem.Int. 18, 263-294.

131. Nishizuka Y. (1986) Science 233, 305-312.

132. Zimmerman U-J.P. and Schlaepfer W.W. (1984) Biochem.Biophys.Res.Comm. 120, 767-774.

133. Adachi Y., Kobayashi N., Murachi T. and Hatanaka M. (1986) Biochem.Biophys.Res.Comm. 136, 1090-1096.

134. Roesler W.J., Vandenbark G.R. and Hanson R.W. (1988) J.Biol.Chem. 263,

9063-9066.

135. Montiminy M.R., Gonzalez G.A. and Yamamoto K.Y. (1990) TINS 13, 184-188.

136. Jungmann R.A., Kelly D.C., Miles M.F. and Milkowski D.M. (1983) J.Biol.Chem. 258, 5312-5318.

137. Hod Y. and Hanson R.W. (1988) J.Biol.Chem. 263, 7747- 7752.

138. Smith J.D. and Liu A.Y-C. (1988) EMBO J. 7, 3711-3716.

139. Miles M.F., Hung P. and Jungmann R.A. (1981) J.Biol.Chem. 256, 12545-12552.

140. Montminy M.R., Sevarino K.A., Wagner J.A., Mandel G. and Goodman R.H. (1986) Proc.Natl.Acad.Sci.USA 83, 6682-6686.

141. Comb M., Birnberg N.C., Seasholtz A., Herbert E. and Goodman H. (1986) Nature (London) 323, 353-356.

142. Silver B.J., Bokar J.A., Virgin J.B., Vallen E.A., Milstead A. and Nilson J.H. (1987) Proc.Natl.Acad.Sci.USA 84, 2198-2202.

143. Collins S., Altschmied J., Herbsman O., Caron M.G., Mellon P.L. and Lefkowitz R.J. (1990) J.Biol.Chem. 265, 19330-19335.

144. Montminy M.R. and Bilezikjian L.M. (1987) Nature (London) 328, 175-178.

145. Roesler W.J., Vandenbark G.R. and Hanson R.W. (1989) J.Biol.Chem. 264, 9657-9664.

146. Phillippe J. and Missotten M. (1990) J.Biol.Chem. 265, 1465-1469.

147. Maekawa T., Sakura H., Kanei-Ishii C., Sudo T., Yoshimura T., Fujisawa J., Yoshida M. and Ishii S. (1989) EMBO J. 8, 2023-2028.

148. Andrisani O. and Dixon J.E. (1990) J.Biol.Chem. 265, 3212-3218.

149. Gonzalez G.A., Yamamoto K.K., Fischer W.H., Karr D., Menzel P., Biggs W., Vale W.V. and Montminy M.R. (1989) Nature (London) 337, 749-752.

150. Latchman D.S. (1990) Biochem.J. 270, 281-289.

151. Yamamoto K.K., Gonzalez G.A., Biggs W.H. and Montminy M.R. (1988) Nature (London) 334, 494-498.

152. MacGregor P.F., Abute C. and Curran T. (1990) Oncogene 5, 451-458.

153. Benbrook D.M. and Jones N.C. (1990) Oncogene 5, 295-302.

154. Imagawa M., Chiu R. and Karin M. (1987) Cell 51, 251-260.

155. DeNoto F.M., Moore D.D. and Goodman H.M. (1981) Nuc.Acids Res. 9, 3719-3730.

156. Cooke N.E. and Baxter J.D. (1982) Nature (London) 297, 603-606.

157. Mitchell P.J., Wang C. and Tjian R. (1987) Cell 50, 847- 861.

158. Jackson R.J. and Standart N. (1990) Cell 62, 15-24.

159. Hata A., Ohno S., Akita Y. and Suzuki K. (1989) J.Biol.Chem. 264, 6404-

6411.

160. Miyake S., Emori Y. and Suzuki K. (1986) Nuc.Acids Res. 14, 8805-8817.

161. Ohno S., Minoshima S., Kudoh J., Fukuyama R., Shimizu Y., Ohmi-Imajoh S., Shimizu N. and Suzuki K. (1990) Cytogenet.Cell Genet. 53, 225-229.

162. Barka T. (1990) Acta Histochem.Cytochem. 23, 245-255.

163. Barrett A.J. (1987) TIBS 12, 193-196.

164. Clemens M.J., (1984) in Transcription and Translation (Hames B.D., Higgins S.J. Ed.s) pp214-215, IRL Press, Oxford.

165. Wallace D.M., (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp33-48, Academic Press, London.

166. Chomczynski P. and Sacchi N. (1987) Anal.Biochem. 162, 156-159.

167. Sambrook J., Fritsch E.F. and Maniatis T. (1989) in Molecular Cloning. A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

168. Jacobson A. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp254-261, Academic Press, London.

169. Davis L.G., Dibner M.D. and Battey J.F. (1986) in Basic Methods in Molecular Biology, Elsevier, London.

170. Ogden R.C. and Adams D.A. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp61-87, Academic Press, London.

171. Williams L.G. and Mason P.J. (1985) in Nucleic Acid Hybridisation. A Practical Approach (Hames B.D., Higgins S.J. Ed.s) pp139-160, IRL Press, London.

172. McMaster G.K. and Carmicheal G.G. (1977) Proc.Natl.Acad.Sci.USA 74, 4835-4838.

173. Amersham Technical Handbook for Hybond-N membranes.

174. Lebrach H., Diamond D. and Boedtker H. (1977) Biochem. 16, 4743-4751.

175. Nucleic Acid Filter Hybridization, Amersham Technical Handbook.

176. Jagus R. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp267-276, Academic Press, London.

177. Kirschbaum B.J., Simoneau J.A., Bar A., Barton P.J.R., Buckingham M.E. and Pette D. (1989) Euro.J.Biochem. 179, 23-29.

178. Jagus R. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp296-304, Academic Press, London.

179. Cobianchi F. and Wilson S.H. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp94-110, Academic Press, London.

180. Chelly J., Kaplan J-C., Maire P., Gautron S. and Kahn A. (1988) Nature (London) 333, 858-860.

181. Kawaski E.S., Clark S.S., Colne M.Y., Smith S.D., Champlin R., Witte O.N.

and McCormick F.P. (1988) Proc.Natl.Acad.Sci.USA 85, 5698-5702.

182. Lee C.C., Wu X., Gibbs R.A., Cook R.G., Muzuny D.M. and Caskey C.T. (1988) Science 239, 1288-1291.

183. Krung M.S. and Berger S.L. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp316-325, Academic Press, London.

184. Saiki R.K., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., Horn G.T., Mullis K.B. and Erlich H.A. (1988) Science 239, 487-491.

185. Promega Technical Laboratory Manual.

186. Miller H. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp145-170, Academic Press, London.

187. Brooks J.E. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp113-129, Academic Press, London.

188. Sanger F., Nicklen S. and Coulson A. (1977) Proc.Natl.Acad.Sci.USA 74, 5463-5467.

189. Parker R.E. (1977) Introductory Statistics for Biology 2nd Edt., Edward Arnold, London.

190. Chirgwin J.M., Przybyla A.E., MacDonald R.J. and Rutter W.J. (1979) Biochem. 18, 5294-5299.

191. MacDonald R.J., Swift G.H., Pryzbyla A.E. and Chirgwin J.M. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, p219-221, Academic Press, London.

192. Berger S.L. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp215-219, Academic Press, London.

193. Poulson R. (1977) in The Ribonucleic Acids 2nd Edt. (Stewart P.R. and Letham D.S.) pp333-367, Springer-Verlag, New York.

194. Dixon R.A.F., Kobilka B.K., Strader D.J., Benovic J.L., Dohlman H.G., Frielle T., Bolanowski M.A., Bennett C.D., Rands E., Diehl R.E., Mumford R.A., Slater E.E., Sigal I.S., Caron M.G., Lefkowitz R.J. and Strader C.D. (1986) Nature 321, 75-79.

195. Wallace R.B. and Miyada C.G. (1987) in Methods in Enzymology (Berger S.L., Kimmel A.R. Ed.s) 152, pp432-442, Academic Press, London.

196. Griffiths S.L., Knowler J.T. and Houslay M.D. (1990) Euro.J.Biochem. 193, 367-374.

197. Dalrymple R.H., Ricks C.A., Baker P.K., Pensack J.M., Gingher P.E. and Ingle D.L. (1984) Poult.Sci. 63, 2376-2383.

198. Davis L.G., Dibner M.D. and Battey J.F. (1986) in Basic Methods in Molecular Biology, pp77, Elsevier, London.

199. Suzuki K., Ohno S., Imajoh S., Emori Y., Kawasaki H. (1985) Biomed.Res. 6,

323-327.

200 Ricks C.A., Dalrymple R.H., Baker P.K. and Ingle D.L. (1984) J.Anim.Sci.59, 1247-1255.

201. Gobet C., Prost E. and Whalen R.G. (1989) Nuc.Acids.Res. 17, 2144.

202. Tindall K.R. and Kunkel T.A. (1988) Biochem. 27, 6008-6013.

203. Bond J.S. and Butler P.E. (1987) Annu.Rev.Biochem. 56, 333-364.

204. Proudfoot N.J. and Whitelaw E. (1988) in Transcription and Splicing (Hames B.D. and Glover D.M.) pp97-129, IRL Press, Oxford.

205. Breitbart R.E., Andreadis A. and Nadal-Ginard B. (1987) Annu.Rev.Biochem. 56, 467-495.

206. Setzer D.R., McGrogan M. and Schimke R.T. (1982) J.Biol.Chem. 257, 5143-5147.

207. Parnes J.R., Robinson R.R. and Seidman J.G. (1983) Nature (London) 302, 449-452.

208. Zehner Z.E. and Peterson B.M. (1983) Proc.Natl.Acad.Sci.USA 80, 911-915.

209. Capetanaki Y.G., Ngai J.,Flytzanis C.N. and Lazarides E. (1983) Cell 35, 411-420.

210. Weiss E.A., Gilmartin G.M. and Nevins J.R. (1991) EMBO J. 10, 215-219.

211. Orlowski M. (1990) Biochem. 29, 10289-10297.

212. Munro H.N. and Fleck A. (1969) in Mammalian Protein Metabolism (Munro H.N. ed.) 3, pp423, Academic Press, London.

213. Smith S.B., Garcia D.K. and Anderson D.B. (1989) J.Anim.Sci. 67, 3495-3502.

214. Wu F.Y., Young C.R., Coleman M.E. and Smith S.B. (1986) J.Anim.Sci. 63, (suppl.1) 237.

215. Alberts B., Bray D., Lewis J., Raff M., Roberts K. and Watson J.D. (1983) in Molecular Biology of the Cell, pp412, Garland Pub.Inc., London.

216. Beerman D.H., Butler W.R., Hogue D.E., Fishell V.K., Dalyrymple R.H., Ricks C.A. and Scanes C.G. (1987) J.Anim.Sci. 65, 1514-1526.

217. Eadara J.K., Dalrymple R.H., Delay R.L., Ricks C.A. and Romos D.R. (1989) Metabolism 38, 883-890.

218. Kim Y.S., Lee Y.B. and Dalrymple R.H. (1987) J.Anim.Sci. 65, 1392-1399.

219. Barnett J.G., Holly R.G. and Ashmore C.R. (1980) Am.J.Physiol. 239, C39-C46.

220. Beerman D.H. (1983) J.Anim.Sci. 57, 328-337.

221. Babij P. and Booth F.W. (1988) Am.J.Physiol. 254, C657-C660.

222. Curran T. and Franza Jnr. R.B. (1988) Cell 55, 395-397.

223. Iwaki K., Sukhatme V.P., Shubeita H.E. and Chiou K.R. (1990) J.Biol.Chem.

265, 13809-13817.

224. Barka T., van der Neon H. and Gresik E.W. (1989) Exp.Cell Res. 185, 419-435.

225. Sassone-Corsi P., Ransone L.R. and Verma I.M. (1990) Oncogene 5, 427-432.

226. Hoeffler J.P., Deutsch P.J., Lin J. and Hasener J.F. (1989) Mol.Endocrinol. 3, 868-880.

227. Adachi Y., Murachi T., Masatoshi M., Ishii K. and Hatanaka M. (1990) Biomed.Res. 11, 313-317.

228. Ilian M.A. and Forsberg N.E. (1990) J.Cell Biol. 111, p371a, Abs.2085.

229. Shaw P.A. and Barka T. (1989) Biochem.J. 257, 685-689.

230. Minty A.J., Caravatti M., Robert B., Cohen A., Daubas P., Weydert A., Gros F.and Buckingham M.E. (1981) J.Biol.Chem. 256, 1008-1014.

231. Minty A.J., Alonso S., Caravatti M. and Buckingham M.E. (1982) Cell 30, 185-192.

232. Reinach F.C. and Fischman D.A. (1985) J.Molec.Biol. 181, 411-422.

233. Reinach F.C., Nagai K. and Kendrick-Jones J. (1986) Nature (London) 322, 80-83.

234. Nagai K. and Thogersen H.C. (1984) Nature (London) 309, 810-812.

235. Ishiura S., Tsuji S., Murofushi S. and Suzuki K. (1982) Biochim.Biophys.Acta 701, 216-223.

236. Etherington D.J.,Taylor M.A.J. and Dransfield E. (1987) Meat Sci. 20, 1-18.